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FMUP FACULDADE DE MEDICINA UNIVERSIDADE DO PORTO

STUDYING THE VALUE OF PROGNOSTIC AND PREDICTIVE BIOMARKERS IN BREAST CANCER PATHOLOGY

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Dissertation to a PhD's Degree in Medicine and Molecular Oncology

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To my daughters, Victória and Valentina

may they overcome the accomplishments of their parents

Ao Avô Polónia, Avó Anita e Tia Bita

(*in memoriam*)

FOREWORD

What is cancer?

Despite being a well-known and widely used word, the precise definition of "neoplasia" is still debatable. One of the most cited definitions of neoplasia belongs to Rupert A. Willis in his famous book "The spread of tumours in the human body" published in 1952: "A neoplasm is an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues and persists in the same excessive manner after cessation of the stimuli which evoked the change." [1].

In the last decades, the study of carcinogenesis has been focused on the somatic mutation theory. According to this theory, cancer has been considered a genetic disease, characterized by sequential accumulation of mutations in key-genes, centring cancer as a cellular problem [2]. However, several studies have showed that the majority of genetic alterations are considered harmless passenger mutations, with no selection advantage [3,4]. Moreover, the context in which the genetic modifications occur are extremely relevant. The associations between genotype and phenotype that are found in one type of cancer cannot be generalized to all cancer types [5]. For instance, studying the effect of targeted therapy in different tumors with the same mutation, it was found that one of the most important determinants of response rate was the histologic diagnosis [6]. Following this line of thought, one of the most amazing set of studies that have been done showing the importance of context in cancer began in the sixties (more than 50 year ago) with experimental pathology. Stevens showed that it was possible to create experimentally teratomas from fetal gonadal ridges transplanted into the testis of adult mice [7]. Later, it was

demonstrated that embryonal carcinoma cells could be inoculated into the mouse blastocyst where they would lose their malignant behavior and assumed normal embryonic cell features into the new chimeric mouse [8]. These experiments show that normal embryonic cells and embryonal carcinoma cells have many common features, providing a link between embryonic development and carcinogenesis.

Later in the eighties, the group of Mina Bissell showed that inoculation of infected cells with Rous sarcoma virus (RSV), a known oncovirus, in chick embryos did not produce malignant tumors as it happens in adult chickens. Instead, these infected cells assumed normal phenotype becoming part of normal embryonic structures. Nevertheless, the same infected cells explanted from these embryos would became transformed in cell culture [9]. These findings also support that context and microenvironment has a profound effect in the malignant transformation of mutated cells.

Summing up, two key facts are taught with these experiments: first, somatic mutations are not required to develop fully malignant tumors and second, malignant behavior can be reprogramed to normal phenotype in the proper context, even in the presence of oncogenic mutations. As such, recently, many authors realize that neoplasia, besides being, literally, the formation of something new, through the proliferation or increased survival of the neoplastic cells, is instead a complex tissue made of several different cell types that establish interactions with each other [10]. The somatic mutation theory is now questioned and challenged, giving origin to different views on cancer initiation and development. For instance, the tissue organization field theory (TOFT) proposes that carcinogenesis takes place at the tissue level of biological organization and that aberrant interactions between different cells and their surroundings are responsible for the formation of cancer [11]. Cancer would not be a cell disease but a tissue disease,

a complex system composed of different cells. Such different cell types include not only the neoplastic cells, but also cells of the microenvironment, like fibroblasts, immune cells, pericytes and even bone marrow-derived cells, among others [12-20]. Far from being considered static elements, the cells of the microenvironment can be recruited to the neoplastic tissue and promote the evolution of this tissue – in other words, normal cells with normal genotype can regulate the malignant behavior of the neoplastic cells. These different theories should not be regarded individually but instead complementing each other, resulting in an extraordinary complex theory of carcinogenesis [21].

As a complex tissue that does not respect the boundaries of normal tissue architecture, neoplasia is also regulated by non-cellular elements of the microenvironment which carry autocrine and paracrine signals. These signals that control normal and neoplastic tissue architecture imbalance (cell number and position) are transmitted from one cell to the other, through the stroma, in a very tightly regulated fashion, in which variations over time are a very important factor. Although, the effects of the non-cellular elements and their temporal variations are very difficult to access experimentally, the mechanisms involved in the maintenance of architecturally complex tissues should be investigated to achieve a more precise knowledge on neoplasia behavior [10,22,23].

Recently, the characterization of the genomes of neoplastic cells microdissected from different areas of the same tumor has revealed intratumoral genetic heterogeneity, the recognition of which has relevant implications in cancer therapy as well as in the establishment of cancer study strategies [24,25]. Accordingly, it is no longer sufficient to understand the biology of neoplasia just to keep the study focus on neoplastic cell genome but through the study of the cross-talk between neoplastic cells and their microenvironment [26]. Moreover, the architectural

relationship of the different sub-clones in the neoplastic tissue might be important to the understanding of the disease, although this study is a very difficult one if we are considering the evaluation of several sub-clones.

Using breast cancer as a model, we studied the role of tumor-based biomarkers in prognostication, exploring heterogeneity and tumor microenvironment. The study of morphology and the quantification of immunohistochemistry and in situ hybridization in different series of breast cancer cases, allowed us to correlate the status of human epidermal growth factor receptor 2 (HER2) and programmed cell death-ligand 1 (PDL1) in breast cancer tissue, as well as the presence of stromal tumor-infiltrating lymphocytes (TILs) in the microenvironment, with clinicopathological features and patient´s outcome. In the end, we identify solutions to clinical-based problems and attempted to shed some light in breast cancer carcinogenesis, hoping to help finding new directions for cancer research.

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LIST OF PUBLICATIONS

1 - Polonia A, Leitao D, Schmitt F. Application of the 2013 ASCO/CAP guideline and the SISH technique for HER2 testing of breast cancer selects more patients for anti-HER2 treatment. *Virchows Arch.* 2016;468(4):417-423
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ABBREVIATIONS

AKT – AKT serine/threonine kinase

ALDH1 – aldehyde dehydrogenase 1

ALK – ALK (anaplastic lymphoma kinase) receptor tyrosine kinase

APC – antigen presenting cell

ASCO – American Society of Clinical Oncology

ATM – ATM (ataxia telangiectasia mutated) serine/threonine kinase

BC – breast cancer

BCS – breast conserving surgery

BFB – breakage-fusion-bridge

BMI – body mass index

BRAF – B-RAF proto-oncogene, serine/threonine kinase

BRCA1 – BRCA1, DNA repair associated (breast cancer 1, early onset)

BRCA2 – BRCA2, DNA repair associated (breast cancer 2, early onset)

CAP – College of American Pathologists

CCND1 – cyclin D1

CD – cluster of differentiation

CDH1 – cadherin 1 (E-cadherin)

CDK4 – cyclin dependent kinase 4

CD49f – integrin subunit alpha 6 (ITGA6)

CEP17 – centromere enumeration probe 17

CI – confidence interval

CIS – carcinoma *in situ*

CSC – cancer stem cell

DAB – 3,3´-diaminobenzidine

DCIS – ductal carcinoma *in situ*

DFS – disease-free survival

dMMR – mismatch repair deficiency

DNA – deoxyribonucleic acid

EGFR – epidermal growth factor receptor (ERBB1 – erb-b2 receptor tyrosine kinase 1)

EMT – epithelial-mesenchymal transition

ER – estrogen receptor

FFPE – formalin-fixed paraffin-embedded

GATA3 – GATA binding protein 3

GH – genetic heterogeneity

GRB7 – growth factor receptor bound protein 7

HER – human epidermal growth factor receptor (ERBB – erb-b2 receptor tyrosine kinase)

HER2 – human epidermal growth factor receptor 2 (ERBB2 – erb-b2 receptor tyrosine kinase 2)

H&E – hematoxylin and eosin

IBC – invasive breast cancer

ICGC – International Cancer Genome Consortium

IHC – immunohistochemistry

INPP4B – inositol polyphosphate-4-phosphatase type II B

ISH – *in situ* hybridization

k – kappa

kDa – kilodalton

Ki-67 – MKI67 (marker of proliferation Ki-67)

MAP3K1 – mitogen-activated protein kinase kinase kinase 1

MAPK – mitogen-activated protein kinase

MDM2 – MDM2 proto-oncogene (mouse double minute 2)

MEK – mitogen-activated protein kinase kinase (MAP2K)

METABRIC – Molecular Taxonomy of Breast Cancer International Consortium

MIEN1 – migration and invasion enhancer 1

MSI – microsatellite instability

MWU – Mann-Whitney *U* test

MYC – MYC proto-oncogene, bHLH transcription factor

NAC – neoadjuvant chemotherapy

NC – not computed

NCB – needle-core biopsy

NCCN – National Comprehensive Cancer Network

NGS – Nottingham grading system

NK – natural killer

NS – not significant

NSCLC – non-small cell lung carcinoma

NST – no special type

OS – overall survival

pCR – pathologic complete response

PCC – Pearson´s correlation coefficient

PD1 – programmed cell death protein 1 (PDCD1)

PDL1 – programmed cell death-ligand 1 (CD274/PDCD1LG1)

PDL2 – programmed cell death-ligand 2 (CD273/PDCD1LG2)

PGAP3 – post-GPI attachment to proteins 3

PgR – progesterone receptor

PI3K – phosphatidylinositol 3-kinase

PIK3CA – phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha

PNMT – phenylethanolamine N-methyltransferase (PENT)

PTEN – phosphatase and tensin homolog

RAS – RAS type GTPase family

RB1 – RB transcriptional corepressor 1

RCC – renal cell carcinoma

RSV - Rous sarcoma virus

SCCHN – squamous cell carcinoma of the head and neck

SISH – silver *in situ* hybridization

SPSS – Statistical Package for the Social Sciences

STAT – signal transducer and activator of transcription

STK11 – serine/threonine kinase 11

TCAP – titin-cap (telethonin)

TCR – T-cell receptor

Tfh – T follicular helper cell

Th – T helper cell

TILs – tumor infiltrating lymphocytes

TNBC – triple-negative breast cancer

TOFT – tissue organization field theory

TOP2A – DNA topoisomerase II alpha

TP53 – tumor protein p53

UK – United Kingdom

WHO – World Health Organization

ABSTRACT

Breast cancer is the most commonly diagnosed cancer in women, responsible for nearly one-third of all new cases diagnosed every year. The probability of developing breast cancer is between 10% and 15%, continuously increasing throughout life, being the main cause of cancer-related death in women. Fortunately, more than half of breast cancer cases are diagnosed in a localized stage, which have a very high survival rate (more than 95%). Nevertheless, breast cancer is a heterogeneous group of lesions, comprising cancers with very different clinical outcomes. The overall goal of this work was to improve the value of prognostic and predictive biomarkers in breast cancer pathology, focusing on the study of human epidermal growth factor receptor 2 (HER2), stromal tumor-infiltrating lymphocytes (TILs) and programmed cell death-ligand 1 (PDL1).

Regarding the study of HER2 in breast cancer, one of the aims was to evaluate the impact of the recent changes introduced on the ASCO/CAP guidelines on the result of HER2 status. A series of primary invasive breast cancer cases was evaluated for HER2 amplification status according to both 2007 and 2013 ASCO/CAP guideline criteria. We observed a significant increase of HER2-positive cases and a decrease of HER2-equivocal cases. Reclassification of the cases from before the introduction of the new ASCO/CAP guideline with the 2013 ISH criteria resulted in an increase of cases with a HER2-positive status and in a decrease of HER2-equivocal cases. In conclusion, the 2013 ASCO/CAP guideline selects more patients for anti-HER2 targeted-therapy, mostly based on the modifications of criteria to evaluate ISH-HER2.

The second aim was to evaluate the intraobserver and interobserver interpretative reproducibility of the HER2 gene amplification assay, by measuring the impact of counting increasing numbers of invasive cancer cells. A cohort of primary invasive breast cancer cases

were evaluated for HER2 gene amplification and the concordance among four observers with different levels of experience was determined. We observed an increase in the intraobserver concordance rate between the first and second evaluations with an increase in cell count. A count of 60 invasive cells was needed to obtain a concordance rate near 95%. The interobserver concordance rate of the HER2 test also increased with the increase in cell count, reaching at least a 90% concordance rate with a count of 60 invasive cells. In conclusion, the minimal cell number recommend in current guidelines should be raised. Moreover, cases with amplification levels close to the threshold should be subjected to a dual count from an experienced observer.

The third aim was to evaluate and compare the HER2 gene amplification *status* in invasive and adjacent *in situ* breast carcinoma and to document the possible presence of HER2 genetic heterogeneity in both components. A cohort of primary invasive carcinomas associated with carcinoma *in situ* were evaluated for HER2 gene amplification. A second cohort of all the cases with HER2 genetic heterogeneity since the introduction of the updated ASCO/CAP HER2 guideline was also characterized, and an evaluation of the HER2 gene amplification in the carcinoma in situ component, if present, was also done. In the first cohort, the HER2 amplification in the invasive carcinoma was positive in 13% of the cases, without the presence of HER2 genetic heterogeneity. All the cases had an associated carcinoma in situ with the same HER2 *status* as invasive carcinoma, with four cases of carcinoma in situ presenting HER2 genetic heterogeneity. In these last cases, two cases presented HER2 gene amplification in the invasive carcinoma. The second cohort included 12 cases with HER2 genetic heterogeneity in a total of 1243 invasive carcinoma cases (0.97%). Additionally, we identified two cases associated with non-amplified carcinoma in situ. In conclusion, HER2 genetic heterogeneity is a rare event

in invasive carcinoma and can already be present in carcinoma in situ, not being an important step in the acquisition of invasive features.

Regarding the study of stromal TILs and PDL1 expression, the aim was to assess the clinical impact of the expression of these biomarkers in breast cancer, namely their correlation with classical pathological features, cancer molecular subtypes, as well as patients' prognosis. Two independent series of invasive breast cancer, one including DCIS pair-matched cases, were selected, and quantification of stromal TILs and expression of PDL1 was determined. In both cohorts evaluated, increased stromal TILs and PDL1 expression were present in about 10% of invasive carcinomas, being significantly associated with each other and both with grade 3 and triple-negative subtype. We observed a similar distribution of stromal TILs and PDL1 expression between carcinoma in situ and invasive carcinoma. Finally, we observed that increased stromal TILs and PDL1 expression were significantly associated with cancer stem cell markers, basal cell markers and vimentin expression. Interestingly, in invasive carcinoma cases with vimentin expression, increased stromal TILs, as well as decreased PDL1 expression, disclosed a better clinical outcome, independently of the main classical breast cancer prognostic factors. In conclusion, we have confirmed the association of stromal TILs and PDL1 expression with aggressive forms of breast cancer and that both are already found in *in situ* stages. We also showed that stromal TILs and PDL1 expression are associated with clinical outcome in cases enriched for a mesenchymal immunophenotype. We also described for the first time a close relationship between cancer stem cell markers and PDL1 expression.

In this thesis, we try to clarify the value of prognostic and predictive biomarkers in breast cancer pathology.

RESUMO

O carcinoma da mama é o carcinoma mais frequente em mulheres, responsável por quase um terço de todos os casos novos por ano. A probabilidade de desenvolver carcinoma da mama é entre 10% e 15%, aumentando continuamente ao longo da vida, sendo a principal causa de morte por cancro em mulheres. Felizmente, mais de metade dos casos são diagnosticados em estádios localizados, que têm uma taxa de sobrevivência muito alta (mais de 95%). No entanto, trata-se de um grupo heterogêneo de lesões, que inclui carcinomas com diferentes prognósticos clínicos. O principal objetivo deste trabalho foi melhorar o valor dos biomarcadores prognósticos e preditivos em patologia mamária, com especial foco no estudo do HER2 (*human epidermial growth factor receptor 2*), dos LITs estromais (linfócitos intra-tumorais) e do PDL1 (*programmed cell death-ligand 1*). Em relação ao estudo do HER2, um dos objetivos foi avaliar o impacto das mudanças recentes introduzidas nas orientações ASCO/CAP sobre o resultado do HER2. Uma série de casos de carcinoma da mama invasivos foi avaliada para o estudo de ampliação de HER2 de acordo com os critérios de orientação ASCO/CAP de 2007 e 2013. Observámos um aumento significativo dos casos HER2-positivos e uma diminuição dos casos equívocos de HER2. A reclassificação dos casos antes da introdução da nova orientação ASCO/CAP com os critérios ISH de 2013 resultou num aumento de casos com status HER2-positivo e numa diminuição dos casos equívocos de HER2. Em conclusão, a orientação 2013 ASCO/CAP seleciona mais pacientes para terapia dirigida anti-HER2, principalmente com base nas modificações dos critérios ISH.

O segundo objetivo foi avaliar a reprodutibilidade interpretativa intraobservador e interobservador do estudo de amplificação do gene HER2, medindo o impacto da contagem de um número crescente de células tumorais. Uma série de casos de carcinoma da mama invasivos

foi avaliada quanto à amplificação do gene HER2 e a concordância entre quatro observadores com diferentes níveis de experiência foi determinada. Observámos um aumento na taxa de concordância intraobservador entre a primeira e a segunda avaliação com o aumento na contagem de células. Foi necessária uma contagem de 60 células invasivas para obter uma taxa de concordância próxima a 95%. A taxa de concordância interobservador do teste HER2 também aumentou com o aumento da contagem celular, atingindo pelo menos uma taxa de concordância de 90% com uma contagem de 60 células invasivas. Em conclusão, o número mínimo de células recomendadas nas orientações atuais deve ser aumentado. Além disso, casos com níveis de amplificação próximos do limiar de positividade devem ser submetidos a uma contagem dupla por um observador mais experiente.

O terceiro objetivo foi avaliar e comparar o estado de amplificação do gene HER2 em carcinoma da mama invasivo e in situ adjacente, documentando a possível presença de heterogeneidade genética HER2 em ambos os componentes. Uma série de carcinomas invasivos primários associados a carcinoma in situ foi avaliada quanto à amplificação do gene HER2. Uma segunda série compreendendo todos os casos com heterogeneidade genética HER2 desde a introdução da nova orientação ASCO/CAP também foi caracterizada, avaliando a amplificação do gene HER2 no componente in situ do carcinoma, se presente. Na primeira série, a amplificação HER2 no carcinoma invasivo foi positiva em 13% dos casos, sem a presença de heterogeneidade genética. Todos os casos apresentaram carcinoma invasivo e in situ com o mesmo status de HER2, com quatro casos de carcinoma in situ apresentando heterogeneidade genética. Nestes últimos casos, dois casos apresentaram amplificação HER2 no carcinoma invasivo. A segunda série incluiu 12 casos com heterogeneidade genética HER2, num total de 1243 casos (0,97%). Além disso, identificámos ainda dois casos associados a carcinoma in situ

não amplificado. Em conclusão, a heterogeneidade genética HER2 é um evento raro no carcinoma invasivo e já pode estar presente no carcinoma in situ, não sendo um passo importante na aquisição de características invasivas.

No que se refere ao estudo dos LITs estromais e da expressão de PDL1, o objetivo foi avaliar o impacto clínico da expressão desses biomarcadores no carcinoma da mama, nomeadamente correlacionar com características anátomo-patológicas clássicas, com os subtipos moleculares de carcinoma, bem como com o prognóstico dos pacientes. Foram selecionadas duas séries independentes de carcinoma da mama invasivo, uma incluindo casos com carcinoma in situ, e foi determinada a quantificação dos LITs estromais e a expressão de PDL1. Em ambas as séries avaliadas, o aumento de LITs estromais e a expressão de PDL1 estavam presentes em cerca de 10% de carcinomas invasivos, estando significativamente associados entre si e ambos com o grau histológico 3 e o subtipo triplo negativo. Observámos uma distribuição semelhante de LITs estromais e expressão de PDL1 entre carcinoma in situ e carcinoma invasivo. Finalmente, observámos que o aumento de LITs estromais e a expressão de PDL1 estavam significativamente associados com os marcadores de células estaminais, marcadores de células basais e expressão de vimentina. Curiosamente, nos casos de carcinoma invasivo com expressão de vimentina, o aumento dos LITs estromais, bem como a diminuição da expressão de PDL1, revelaram um melhor prognóstico clínico, independentemente dos principais fatores prognósticos do carcinoma da mama. Em conclusão, confirmámos a associação de LITs estromais e expressão de PDL1 com formas agressivas de carcinoma da mama e que ambos já se encontram em fases in situ. Também mostrámos que os LITs estromais e a expressão de PDL1 estão associados ao prognóstico clínico em casos com um imunofenótipo mesenquimatoso. Também descrevemos

pela primeira vez uma estreita relação entre os marcadores de células estaminais e a expressão de PDL1.

Nesta tese, tentámos clarificar o valor dos biomarcadores prognósticos e preditivos em patologia mamária.

CHAPTER 1

GENERAL INTRODUCTION

1.1 BREAST CANCER

1.1.1 Epidemiology

The lifetime likelihood of being detected with cancer in the general population is about 40%, being slightly higher for men than for women, making cancer an important public health problem in western world. As such, cancer is the second leading cause of death, following cardiovascular diseases, accounting for about 25% of all deaths, and already the primary reason of mortality in the 5th to the 8th decade of life [27,28].

The most frequent cancers diagnosed in men are prostate, lung and bronchus, and colorectal cancers, representing nearly half of all cancer cases (44%). On the other hand, in women, breast, lung and bronchus, and colorectal cancers are the most common types of cancer, together accounting for about 50% of the cases (Figure 1). Prostate and breast cancers alone are responsible for more than 20% and nearly one-third of new cancer diagnoses in men and women, respectively [27].

Estimated New Cases

Males				Females		
Prostate	180,890	21%		Breast	246,660	29%
Lung & bronchus	117,920	14%		Lung & bronchus	106,470	13%
Colon & rectum	70,820	8%		Colon & rectum	63,670	8%
Urinary bladder	58,950	7%		Uterine corpus	60,050	7%
Melanoma of the skin	46,870	6%		Thyroid	49,350	6%
Non-Hodgkin lymphoma	40,170	5%		Non-Hodgkin lymphoma	32,410	4%
Kidney & renal pelvis	39,650	5%		Melanoma of the skin	29,510	3%
Oral cavity & pharynx	34,780	4%		Leukemia	26,050	3%
Leukemia	34,090	4%		Pancreas	25,400	3%
Liver & intrahepatic bile duct	28,410	3%		Kidney & renal pelvis	23,050	3%
All Sites	**841,390**	**100%**		**All Sites**	**843,820**	**100%**

Figure 1

Leading cancer types for the estimated new cancer cases by sex (from Siegel et al [27])

Although the global cancer incidence rate has been declining since the beginning of the millennium, in women it has remained constant [27]. The decline has been mainly attributed to the decrease of incidence of prostate cancer, as well as colorectal and lung and bronchus cancers in men. The main reasons are due to the falling of routine prostate cancer screening with the PSA test, the introduction of a colorectal cancer screening by colonoscopy, which can detect and remove precancerous lesions, and, lastly, the reduction in smoking habits [27]. However, the increased cancer incidence in women in the 80s was partially due to the increase in lung and breast cancers, reflecting smoking prevalence and deviations in female reproductive patterns as well as the increased detection of asymptomatic disease during mammography screening [27].

In the last decades, it has been observed, in both genders, a constant decrease in overall cancer death rate, most pronounced in men [27]. In women, the decay in overall cancer death rate reflects the decrease in death rates of breast and colorectal cancers, mostly because of developments in early detection through screening programs and improvements in treatment [27]. Therefore, the most frequent causes of cancer death in men are lung and bronchus, prostate and

colorectal cancers whereas in women are lung and bronchus, breast and colorectal cancers (Figure 2) [27].

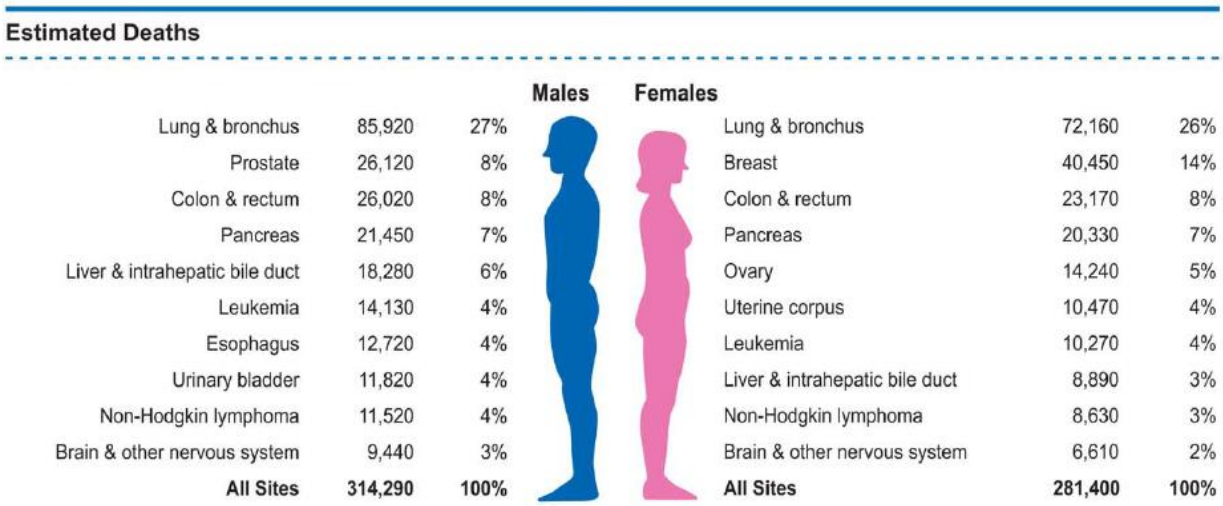


Figure 2

Leading cancer types for the estimated deaths by sex (from Siegel et al [27])

In Portugal, as in several western European countries, breast cancer (BC) is the most commonly diagnosed cancer, representing about 30% of all cancers in women, followed by colorectal, thyroid and gastric cancers, representing nearly 60% of all cancer cases. In men, prostate, colorectal and lung and bronchus cancers are the most frequent cancers diagnosed, together accounting for more than 50% of the cases. Consequently, the main causes of cancer-related death in men are lung and bronchus, prostate and colorectal cancers whereas in women are breast, colorectal and gastric cancers [29].

In women, the probability of developing BC in their lifetime is between 10% and 15%, continuously increasing throughout life [27]. BC is identified at a younger age than other frequent cancers, with a median age of diagnosis at 61 years, with about 20% of the cases between ages 30 and 49 years [30]. Remarkably, in adults younger than 50 years, the probability of developing

cancer is actually higher for women than for men due to the prevalence of breast, genital and thyroid cancers in young women. Concerning the median age of BC death, that is at 68 years, with a mortality rate about 15% of the incidence rate and a wide variation according to the available treatment [27,31]. Notably, BC is the most frequent cause of cancer death in women aged between 20 and 59 years old [27].

Of notice, there are important discrepancies to be considered amongst different ethnicities. For example, black women have slightly lower BC incidence rate than white women, whereas BC death rate is about 40% higher. The precise explanation remains unknown, although probably represents a mixture of biologic and environmental factors, including differences in the molecular subtype and stage at diagnosis, as well as higher prevalence of comorbidities, limited access to care and differences in response to therapy. Regarding the molecular subtype (for details see below), black women have the lowest proportion of luminal A and nearly the double proportion of triple-negative BC, which explains the higher death rate amongst them [31].

More than half of BC cases are diagnosed in a localized stage (about 60%), a third of patients present regional metastases and only less than 10% are detected with distant metastases at diagnosis. Importantly, the 5-year survival rate decreases from more than 95% in localized stages to 85% in regional stages, and to 25% in patients with distant metastases [27].

When a localized BC is diagnosed, breast conserving surgery (BCS), followed by radiation therapy, has the same long-term survival than a total mastectomy [32,33]. Nevertheless, total mastectomy is still required for a proportion of patients with aggressive disease (such as locally advanced stage, large or multiple tumors) or when post-surgery radiation is not possible (such as preexisting medical condition) [34]. Curiously, an increasingly number of women eligible for BCS choose total mastectomy due to fear of recurrence and side effects of radiation therapy [34].

Additionally, in almost 15 years, contralateral prophylactic mastectomy has dramatically increased from 5% to 30% [35].

In the last 40 years, the overall 5-year relative survival rate for BC has improved from about 75% to nearly 90%, mostly attributed to improvements in earlier detection and treatment (such as chemotherapy, hormone therapy and targeted therapy) [36]. The 5-year, 10-year, and 15-year relative survival rates for BC are 89%, 83%, and 78%, respectively [30].

1.1.2 Risk factors

Studies of populations migrating from low- to high-risk areas of developing BC show that the risk for migrant populations increases to similar levels of the host country within two generations, suggesting that environmental factors play an important role in the etiology of BC [37].

Specific environmental factors identified in the development of BC include high-calorie diet, rich in animal fat and proteins, and lack of physical exercise [38,39]. Additionally, alcohol consumption has also been associated with increased risk of BC [40,41]. However, all these factors are associated with lower risk of BC, implying that BC has a multifactorial origin.

More relevant is the unequivocal evidence from epidemiologic studies that both endogenous and exogenous sex hormones contribute to the origin of BC [42]. Reproductive risk patterns include early menarche, nulliparous, late motherhood, fewer offspring and late menopause [27]. Additionally, breast feeding for at least four months is usually related with a decrease risk of developing BC [43-45]. Additionally, oral contraceptives were thought to increase the risk of BC due to the presence of estrogen and progestin (a synthetic progestogen similar to progesterone) [46]. However, recent studies have shown that the risk of developing BC associated with the use of

oral contraceptives is very small, if any, even in women who have been using it for prolonged periods of time [47,48]. Regarding hormone-replacement therapy in postmenopausal women, researchers observed a significant link between unopposed estrogen therapy and risk of BC, mainly among leaner women with the longest duration of its use [43,49]. Still, the above therapy is usually given with the addition of a progestin, which decreases the increased risk of endometrial carcinoma associated with the use of unopposed estrogen regimens. Nevertheless, not only the impact of progestin does not have a BC protective effect, as the relative risk of BC for users of estrogen-plus-progestin combinations was significantly higher than for users of estrogen-only preparations [43,49,50].

Although body mass index (BMI) is also positively associated to risk of postmenopausal BC, it is inversely associated to risk of premenopausal BC [51]. In fact, overweight and obesity is thought to be responsible for about 10% of cases of postmenopausal BC [37]. Additionally, weight loss after menopause is linked with a decreased risk of BC [52]. The reduced risk of premenopausal BC in heavier women is probably related to the irregular menstrual cycles and increased anovulatory cycles, which reduces the cumulative exposure to sex hormones and, consequently, the risk for hormone sensitive tumors [53]. Moreover, obesity is linked with increased levels of insulin and related growth factors, which can increase the risk for some BC subtypes [54,55].

Finally, genetic susceptibility also impacts on the risk of BC, with near 10% of BC cases being triggered by inherited germline mutations [5]. Most familial BC arises in the background of the hereditary breast-ovarian cancer syndrome (about 90%), which is caused by germline mutations in tumor suppressor genes (BRCA1 and BRCA2) in an autosomal dominant manner with variable penetrance, with tumors showing somatic loss of the second allele. Interestingly, BRCA1 and BRCA2 BCs are very different regarding their expression of hormone receptors.

BRCA1 BCs are usually estrogen receptor (ER) negative, whereas BRCA2 are typically ER positive [56,57]. Additionally, there are several inherited syndromes in which BC can be a manifestation, which include Li-Fraumeni syndrome (TP53 mutation), Cowden syndrome (PTEN mutation), Peutz-Jeghers syndrome (STK11 mutation), hereditary diffuse gastric cancer syndrome (CDH1 mutation) and ataxia-telangiectasia syndrome (ATM mutation) [5].

Evaluation for hereditary BC predisposition is an essential component of the clinical BC management. Considering just features that a pathologist can identify, according to NCCN guidelines, germ line testing for BRCA should be recommended in patients with: BC at age ≤ 45 years, multiple BC at age ≤ 50 years, triple-negative BC at age ≤ 60 years, male BC and ovarian cancer. Additionally, germ line testing for TP53 should also be recommended in BC at age ≤ 31 years [58].

The identification of a germline mutation can increase the screening and treatment of the patient and their relatives, which can take risk-reducing salpingo-oophorectomy and bilateral mastectomy, procedures known to increase life expectancy [5].

1.1.3 Histological classification and grading

Invasive BC is a heterogeneous group of lesions and the basis for its classification includes the evaluation of histological criteria mostly based on morphology (architectural patterns and cytological features). Classical pathological features assessed such as histological type and grade, tumor size and axillary lymph-node involvement are known to correlate with clinical prognosis and, along with immunohistochemical analyses, provide the basis for therapeutic selection performed by clinicians [59,60].

According to the World Health Organization (WHO) classification, the most frequent type of invasive BC is invasive carcinoma of no special type (NST) representing about 75% of the cases [37]. Regarding special subtypes, the most common is the invasive lobular carcinoma (Figure 3A), accounting for 5% to 15% of the cases, and the remaining 10% to 20% of the cases incorporates more than 15 special subtypes of BC, some of which associated to an excellent prognosis, such as tubular, mucinous and papillary encapsulated carcinomas (Figure 3B to 3D, respectively), and others with unfavorable prognosis, like metaplastic or invasive micropapillary carcinomas (Figure 3E and 3F, respectively). As such, BC histologic classification has been shown to be an important prognostic factor correlating with long term survival [61].

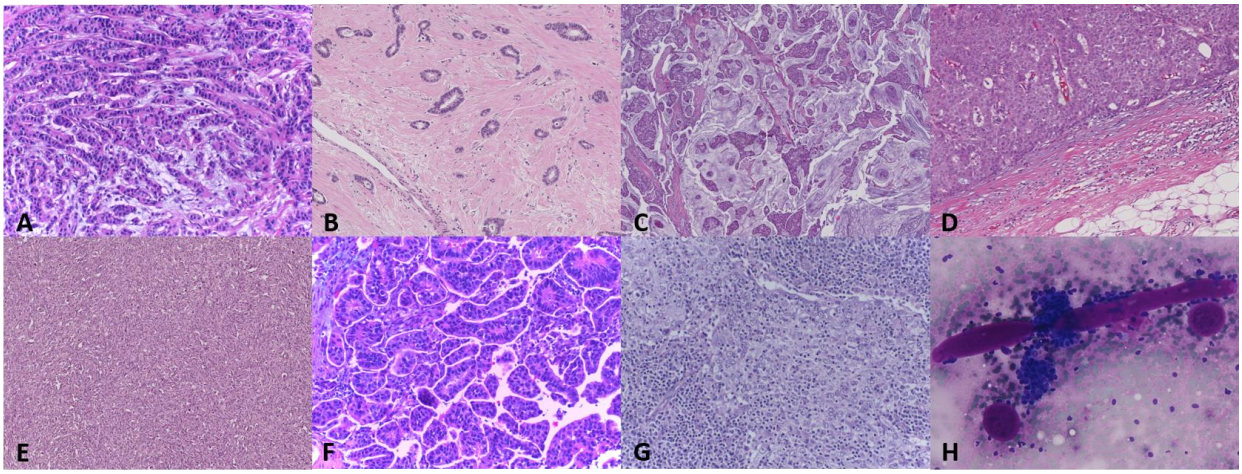


Figure 3
Examples of special BC histologic subtypes

A: lobular carcinoma; B: tubular carcinoma; C: mucinous carcinoma; D: papillary encapsulated carcinoma; E: metaplasic carcinoma; F: micropapillary carcinoma; G: medullary carcinoma; H: adenoid cystic carcinoma (from personal cases)

All invasive breast carcinomas should be graded according to the Nottingham grading system (NGS – Elston-Ellis modification of Scarff-Bloom-Richardson grading system) [62]. NGS is based

on a semi-quantitative evaluation of three morphological characteristics: the amount of glandular and tubular differentiation, the degree of nuclear atypia and the mitotic rate. Each feature is assessed independently in a scoring system of 1 to 3, and the scores are added to produce a grade. Glandular and tubular differentiation is evaluated over the whole tumor in a low-power view, where only structures with central lumina bordered by polarized cells are accepted. Nuclear atypia is assessed in the area with tumor cells presenting a high degree of pleomorphism, by comparing the regularity of nuclear size and shape with nuclei of epithelial cells in benign adjacent breast parenchyma. Lastly, mitotic rate is counted in an area showing the most proliferation activity, involving the standardization to a fixed field area and is depicted as the total number of mitotic figures in 10 high-power fields (HPF). Only clearly identifiable mitotic figures should be accepted, excluding hyperchromatic, karyorrhectic or apoptotic nuclei. At last, the final grading corresponds to the addition of the three individual scores: Grade 1 (score 3 to 5), Grade 2 (score 6 or 7) and Grade 3 (score 8 or 9) (Figure 4) [37].

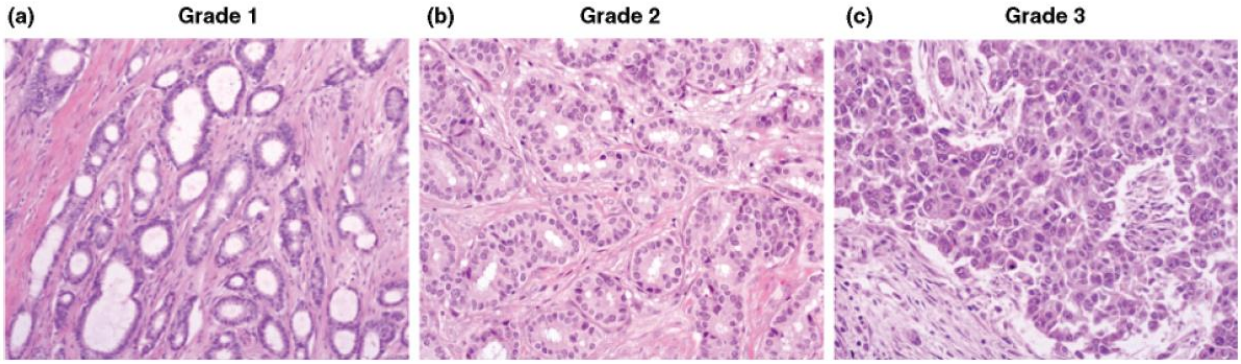


Figure 4

Histological grade of breast cancer as assessed by the Nottingham Grading System (from Rakha et al [59])

Numerous studies have shown that histological grade is an independent prognostic factor in BC, equivalent to that of lymph node status and even greater than that of tumor size [63].

Furthermore, low-grade and high-grade tumors show distinct molecular profiles at the genomic and transcriptomic levels, suggesting that they probably represent distinct diseases [64]. Additionally, it has been demonstrated a significant association between histological grade and survival of patients with invasive BC [59,65]. Several studies applying molecular signatures showed that histological grade is an independent prognostic factor for ER-positive BC [66].

Histological grade should also be performed in needle-core biopsy (NCB) samples because it shows good concordance rate with surgical excision specimens [59,67]. Typically, the discordant cases are due to the decreased ability to evaluate mitotic activity on NCB, underestimating the true histological grade of the tumor [67,68]. As such, the upgrade is usually seen in grade 1 or 2 in the NCB to grade 2 or 3 in the surgical specimen, respectively. Despite these limitations, evaluation of histological grade on NCB provides predictive information for neoadjuvant chemotherapy, independently of the type of regimen used for the treatment [69].

One of the critics towards NGS is the suboptimal inter- and intra-observer concordance rate, with most of the studies showing moderate agreement, especially in grade 2 tumors [59]. It is well known that histological grading is dependent on pre-analytical parameters, such as ischemic time and fixation time, and that its impairment usually compromises the evaluation of the mitotic count, one of the features evaluated in NGS, leading to a downregulation of the grade [59]. Fortunately, the application of optimized and standardized methods can provide an improvement of agreement rates in histological grading.

1.1.4 Molecular classification

More than 15 years ago, gene expression profiling through microarray analysis provided a molecular classification of invasive BC with distinct clinical outcomes and response to therapy

[70]. Hierarchical cluster analysis included genes that varied more in expression between different tumors than between repeated samples from the same tumor (known as intrinsic genes). The subsequent molecular classification revealed two important BC subgroups: ER positive and ER negative (Figure 5). Each subgroup is composed of two intrinsic subtypes, namely luminal A and B, and human epidermal growth factor receptor 2 (HER2)-overexpressing and basal-like (or triple-negative), respectively [71-73]. In general, about three quarters of BC are classified as luminal A, about 10% as basal-like and luminal B each, and about 5% as HER2-overexpressing [31].

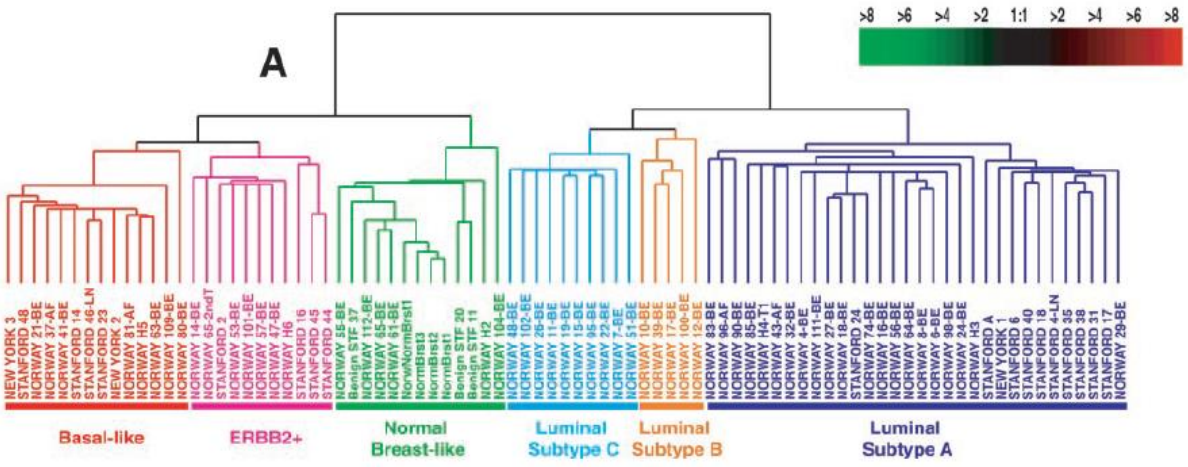


Figure 5

Gene expression patterns analyzed by hierarchical clustering (from Sorlie et al [72])

Luminal BC has a bimodal age distribution with main late modes just below age 70 years and minor modes around age 50 years, whereas basal-like BC has a predominant early-onset mode near age 50 years. Curiously, the age distribution of HER2-positive BC lays between the luminal and basal-like BC. Regarding BC-specific death, luminal A BC have a relatively constant death rate of about 2% every year after BC diagnosis, while luminal B, HER2-positive and basal-like

present a death rate peak around 8% per year two years after initial diagnosis and then a strong decline [74].

Usually, luminal A BC are smaller, with lower histological grade and less likely to have lymph node metastases [43]. Consequently, it is associated with higher survival rates mostly due to the predictive response to hormonal therapy [75]. On the contrary, luminal B, HER2-overexpressing and basal-like are more often high grade with more frequent lymph node and distant metastases [43].

The complexity of BC is not entirely understood just by examining the histopathology features and the gene expression profiling [76]. The characterization of structural DNA changes by array comparative genomic hybridization and next-generation sequencing analysis has provided additional prognostic information [77,78].

The METABRIC group (Molecular Taxonomy of Breast Cancer International Consortium) has characterized the genomic and transcriptomic architecture of near 2000 BCs to evaluate the impact of genomic changes on the transcriptome and were able to identify 10 new molecular subgroups (integrative clusters) each associated with distinct genomic aberrations, gene expression profiling and clinical outcome (Figure 6) [76,79].

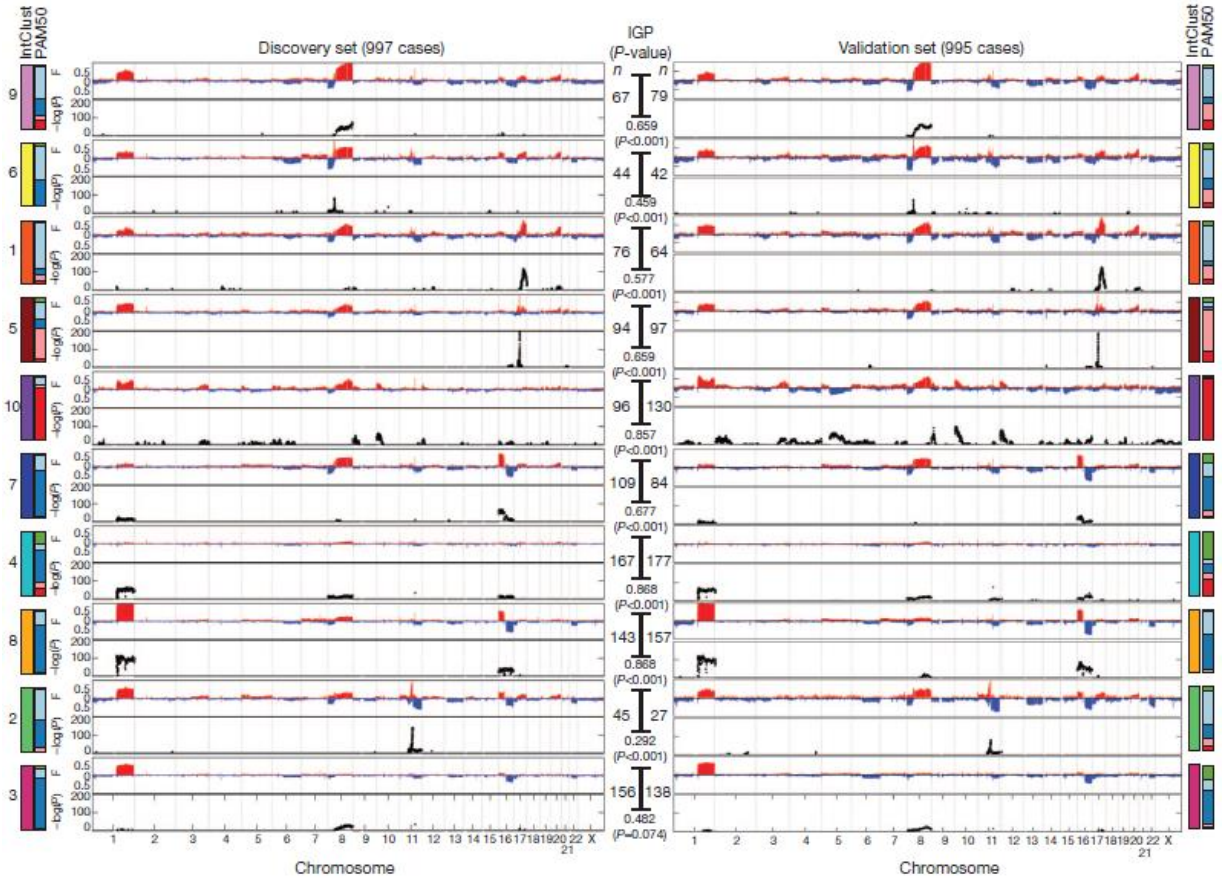


Figure 6

The integrative subgroups (from Curtis et al [79])

Briefly, in this classification, the 3 subgroups with better clinical prognosis (integrative cluster 3, 7 and 8) included predominately luminal A cases with low histological grade, resulting in a disease-specific survival rate at 10 years of 80-90%. Interestingly, the METABRIC group also identified a specific subgroup of BC (integrative cluster 4) composed by both ER-positive and ER-negative cases (including basal-like) associated with favorable prognosis with a disease-specific survival rate at 10 years near 80%. Several of these cases displayed abundant lymphocytic infiltration, low levels of genomic instability and about 20% revealed deletions at the T-cell receptor (TCR) loci. Finally, it was possible to distinguish a subgroup of luminal A and B tumors (integrative cluster 2) associated with the worst disease-specific survival rate at 10

years of ER-positive cases (about 50%). The genetic alterations apparently responsible for the aggressive behavior were the relatively high levels of genomic instability of these tumors and the amplification of 11q13/14 which includes genes involved in cell-cycle regulation (such as *CCND1* – cyclin D1) [76].

Luminal BC cases are the most heterogeneous regarding gene expression profiling, mutation range, copy number changes and clinical outcomes. The most frequently genomic alterations in these tumors include mutations in *PIK3CA*, *TP53*, *GATA3* and *MAP3K1* genes, amplifications of *CCND1*, *MDM2* and *CDK4*, as well as deletions of *PTEN* and *INPP4B* [78]. Besides *HER2* gene amplification, the most commonly genomic alterations in HER2-positive BC include mutations in *TP53* and *PIK3CA* genes, amplifications of *CCND1*, *MDM2* and *CDK4*, as well as deletions of *PTEN* and *INPP4B* [78]. The overlap of genomic alterations between HER2-positive and luminal BC cases comes from the fact that the former is composed of both ER-negative and ER-positive cases in almost evenly amounts [76]. Finally, the most frequently genomic alterations in basal-like BC include mutations in *TP53* gene, amplifications of *MDM2* and *MYC*, as well as deletions of *PTEN*, *INPP4B* and *RB1* [78].

Gene expression profiling analysis has also been applied to carcinoma in situ (CIS), which identified the same intrinsic subtypes as the molecular classification described for invasive BC and even allowing discrimination between low-grade and high-grade CIS [80,81]. Remarkably, CIS preferentially cluster with invasive lesions of similar grade, implying that CIS are precursors of invasive BC with similar grade progressing along different pathways [82]. Additionally, molecular heterogeneity from gene expression analysis has also shown that there are more differences between separate molecular subgroups than between in situ and invasive lesions within a subgroup [83].

Lastly, while invasive carcinomas of NST and invasive lobular carcinomas comprise different molecular subtypes, the remaining histological special types are very homogeneous, belonging predominantly to one molecular subtype, supporting the existence of these histological specific entities. Furthermore, it was also showed that basal-like tumors usually associated with poor prognosis included histological special types with favorable outcome, such as medullary and adenoid cystic carcinomas (Figure 3G and 3H, respectively) [84].

Comparative studies have shown that different molecular tests evaluate distinct groups of genes, the majority of which with little overlap amongst them, and that different algorithms used for molecular classification create unreliable results, achieving only moderate agreement rates [85]. Curiously, although several thousands of genes can be differentially expressed between different tissues, any randomly selected set of genes that are large enough (as few as 100 genes) can mimic the classification achieved with the full set of genes [86]. Interestingly, only basal-like subtype reveals high concordance rate when compared with different molecular tests, revealing that the other subtypes are critically dependent on the applied algorithm and that the results from different studies should not be generalized.

Concerning HER2 subtype, the problem of molecular classification has additional clinical implications. Cases classified as HER2-positive by FDA-approved methods (immunohistochemistry (IHC) and in situ hybridization (ISH)) can be classified as different molecular subtypes by gene profiling analysis in a considerable proportion of cases [85]. As such, the molecular classification is not equivalent to the clinical identification of HER2-positive BC and should not be used to select patients for targeted therapy.

As mentioned before, HER2-positive BCs are heterogeneous, including both ER-positive and ER-negative cases, with significant differences on their response to targeted therapies. In fact,

various clinical trials have shown that the variability in response to trastuzumab depends on ER status of the tumors [87-89]. The Breast Cancer Working Group from the International Cancer Genome Consortium (ICGC) studied a series of HER2-positive BC cases, identifying 4 groups (A to D) of gene expression, each with typical genomic alterations. The first two (A and B) were mainly composed of ER-positive and luminal B subtypes, whereas the last two (C and D) were typically composed of ER-negative and HER2-enriched subtype. Notably, group A did not show TP53 mutations while group D lacked PIK3CA mutations, in comparison with the other groups [90]. Considering all these molecular studies, HER2-positive BC rather than represent a specific oncogenic pathway, is just a subtype characterized by a specific gene amplification that can appear in both luminal (ER-positive) and basal (ER-negative) BC progenitor cells.

Additionally, the study could characterize more accurately the HER2 amplicon region, which included 6 genes: TCAP, PNMT, PGAP3, MIEN1, GRB7 and, evidently, HER2 [90]. Interestingly, the genomic alterations present in various tumors were consistent with a breakage-fusion-bridge (BFB) cycle. BFB is a DNA amplification mechanism that initiates with a double-strand break followed by the fusion of the two loose ends of the sister chromatids during replication. During mitosis, in the next anaphase, the centromere breaks and sister chromatids are pulled in the opposite direction forming a bridge as the ends are fused and a new break is induced at a random location, perpetuating the cycle. Consequently, at each cycle, segments of DNA are unevenly inherited by daughter cells leading to a population of cells with heterogenous copy numbers. As such, genomic segments amplified by BFB mechanism display two signatures: fold-back inversions in the region containing breakpoints (palindromic sequences) and copy number heterogeneity [91]. Nevertheless, not all cases were expected to have occurred through a BFB

cycle, and different gene amplification mechanisms, like the formation of double-minutes chromosomes, could also be involved in the formation of HER2-positive BC [90].

Additional to the identification of the molecular intrinsic subtypes, several studies of gene expression profiling have also identified groups of genes that are able to provide prognostic and predictive information on BC patients. Numerous studies have found similar prognostic performances between different molecular signatures (in around 80% of the cases), suggesting robustness across several genomic platforms, especially regarding poor outcome subtypes (such as basal-like, HER2-overexpression and luminal B) [92,93]. Not unexpectedly, it is among luminal A tumors that greater variability in the outcome has been found [92]. Currently, according to ASCO guidelines, clinicians may use the 21-gene recurrence score (Oncotype DX), the 12-gene risk score (EndoPredict), the Prediction Analysis of Microarray-50 (PAM50) risk of recurrence score and the Breast Cancer Index to guide decisions on adjuvant systemic chemotherapy only in ER-positive, HER2-negative and node-negative BC patients. In the remaining situations (ER-negative, HER2-positive and node-positive BCs), these molecular signatures should not be used in the above setting [94].

Finally, all the components within a tumor sample (neoplastic and non-neoplastic cells) contribute to the expression profiling analysis and the relative amount of these elements have significant effects on the final molecular classification [95]. As such, the contamination with normal breast parenchyma, proliferating stromal cells or inflammatory infiltrate can switch the molecular classification based on the evaluation of ER- and proliferation-related genes. This weakness probably accounts for the existence of a “normal breast-like” cancer in molecular profiling, not mentioned earlier, which disappears after microdissection of the tumor samples [85,96].

1.1.5 Prognostic and predictive biomarkers

The current cancer care guidelines for BC recommend that estrogen receptor (ER), progesterone receptor (PgR) and human epidermal growth factor receptor 2 (HER2) *status* must be routinely determined in all patients with invasive BC, BC recurrences and BC metastases [75,97]. These guidelines were published to improve laboratory performance in the determination of these markers, which provide useful predictive information regarding response to targeted therapy.

The importance of measuring ER expression in BC samples derives from the fact that ER-positive BCs, representing about 75-80% of all BC cases, benefit from endocrine therapy whereas ER-negative BCs do not [98-100]. Early studies suggested that patients with ER-positive/PgR-negative BC could have a worse prognosis than patients with ER-positive/PgR-positive BC, probably because PgR expression is estrogen dependent and, thus, would serve as a marker of an undamaged estrogen response pathway [101,102]. Nevertheless, patients with ER-positive/PgR-negative BC should not be excluded from endocrine therapy, nor the curious ER-negative/PgR-positive BC cases [100].

The same principle applies to HER2 expression, where numerous clinical trials have demonstrated that HER2-targeted therapy given during and/or after chemotherapy improves progression-free survival and overall survival only in patients with BC disclosing HER2 gene amplification/protein overexpression [103-106]. For this reason, HER2 is a helpful marker for therapy decision making in patients with BC and the accurate assessment of HER2 status identifies patients who are most likely to benefit from targeted therapy.

Gene expression profiling studies have revealed that the prognostic impact of the gene signatures derives mainly from the proliferation-related genes. Interestingly, when these signatures are split into ones with and without proliferation-related genes, only the gene signatures with proliferation-related genes maintain prognostic information, sometimes even improving their performance [93].

One of the most frequent assessments of cell proliferation in formalin-fixed paraffin-embedded (FFPE) tumor samples consists in the evaluation by IHC of Ki67 antigen, a nuclear protein expressed in all phases of the cell cycle other than G0 phase [107].

In fact, the St Gallen BC consensus allows the discrimination of ER-positive tumors between luminal A-like (low proliferation and better prognosis) and luminal B-like (higher proliferation and worse prognosis) BCs by IHC testing, although the use of Ki67 should be interpreted considering the knowledge of the local laboratory values and the fact that significant discordance can exist between the gene-based and IHC-based expression profiles for the classification of tumors into the molecular intrinsic subtypes [60].

Nevertheless, although IHC measurements of proliferative activity using the Ki67 antibody convey prognostic information, as well as the high Ki67 values predict the benefit of adding chemotherapy to BC patients, the identification of suitable cutoffs for clinical management has been a challenging task [108,109]. The main problem derives from both pre-analytic and analytic methodologic variations. First, there is few data on the effects of ischemic time, duration of fixation and antigen retrieval in the staining of Ki67; second, there is a lack of consensus, despite the existence of international guidelines, on which part of the tumor should be scored (periphery of the tumor, hot spots or overall average), which introduces significant variability across laboratories [110,111].

The Breast Cancer Working Group has already provided guidelines for analysis and reporting Ki67 expression [111]. After the implementation of the guidelines, which aimed to reduce preanalytical and analytical variations in the evaluation of Ki67, a reproducibility study of Ki67 in BC among experienced pathology laboratories from North America and Europe, with a record of publishing literature regarding the clinical usefulness of Ki67, was performed. The study found high intra-laboratory reproducibility but only moderate inter-laboratory reproducibility of Ki67 expression in BC making its use difficult in clinical decision-making settings [110]. Interestingly, the laboratories that showed highest intra-laboratory reproducibility were the ones with the highest inter-laboratory reproducibility, which preferentially used formal counting methods rather than visual estimation measurements [110].

For all the reasons mentioned above, routine testing of BC samples for Ki67 expression is optional and not currently recommended by either the College of American Pathologists (CAP) or the American Society of Clinical Oncology (ASCO) or the National Comprehensive Cancer Network (NCCN) guidelines [94,112]. Nevertheless, the lack of assess to molecular tests in clinical practice, only available in international reference centers and still expensive, supports the request by oncologists for Ki67 evaluation in specific cases to assist in treatment decision scenarios.

Therefore, in clinical practice, IHC can be used to classify BC cases into the molecular intrinsic subtypes routinely evaluating ER, PgR, HER2 and Ki67 [60]. This approach to molecular classification has been shown to be clinically useful [113,114]. Accordingly, BC cases can be subdivided into luminal tumors which are recognized by the presence of ER and/or PgR expression [115]. Moreover, proliferation markers, such as Ki-67, can sub-classify luminal tumors into luminal A (low-proliferation) and luminal B (high-proliferation) cases [116]. Luminal B tumors also include cases with both hormone-receptor and HER2 positivity. The ER-negative subgroup

can be additionally split based on the expression of HER2 giving origin to HER2-positive and triple-negative BC (TNBC), usually also expressing basal cell markers.

Remarkably, no international consensus still exists regarding which basal cell markers should be used to define basal-like tumors [117]. The suggested IHC markers include the expression of high-molecular-weight cytokeratins (CK5/6, CK14 or CK17), epidermal growth factor receptor (EGFR) and p-cadherin [118-120].

Although the resemblance of TNBC and basal-like BC are striking, these two nomenclatures are not identical, even though it has been used synonymously. Nevertheless, most TNBC express basal-like phenotype in gene expression profiling (around 70%) and most basal-like tumors are negative for ER, PgR and HER2 (near 80%) [121].

In the same way, almost 25% of clinically ER-positive BCs are classified by gene expression profiling as non-luminal BC and almost half of clinically HER2-positive BCs are not classified as HER2-overexpression by molecular classification [96,122]. Interestingly, when molecular profiling analysis is compared to current clinicopathological characteristics plus IHC routinely used, it has been shown that the difference in prognostic information in BC patients is minimal, with most of the benefit limited to ER-positive tumors [123].

Other biomarkers have been recently studied in BC, which include cancer stem cell (CSC) markers. CSCs are responsible for tumor growth, progression and metastasis due to their stem cell-like features: self-renewal and aberrant differentiation.[124] Additionally, it has been shown that CSCs are more resilient to several types of treatment, such as radio and chemotherapy [125-127]. In BC, CD44 was found to be associated with stem cell-like features and CD24 with differentiated epithelium.[128] Consequently, the combination of these cell surface markers

($CD44^{+}/CD24^{-/low}$) was considered a marker of CSCs.[129-131] Additionally, CD49f and ALDH1 have also been demonstrated as CSC markers, being associated to worse prognosis.[132-135]

Within these biomarkers, and including some more, some have shown independent prognostic information, and much more will certainly be studied in the future. In this introduction, we chose to mention the biomarkers that are currently mandatory in the evaluation of BC cases according to the most updated international guidelines, as well as the ones that are relevant to understand this thesis. Nonetheless, the major limitation to the inclusion of a new biomarker in the clinical practice, despite the obvious increase in the cost of the pathology examination, is the usual absence of predictive information regarding current BC treatment. The biomarkers that prove to add predictive information, specially related to targeted therapy, will immediately be introduced in the clinical practice.

1.2 HER2 oncogene

HER2 (ERBB2, formerly HER2/neu, c-erbB2) is a member of a family of 4 transmembrane protein receptors with tyrosine kinase activity (human epidermal growth factor receptor 1 to 4 - HER1 to HER4) [136]. This 185 kilodalton (kDa) glycoprotein had several names throughout time as it was being identified in several types of cells. The name Neu appeared because it was originally identified in ethylnitrosourea-induced rat neuroblatomas. In addition, the human gene was defined as ErbB-2 due to its homology with ErbB (avian erythroblastosis oncogene B) [137].

The binding of a ligand to the extracellular domains of HER proteins promote a conformational change that will allow its dimerization and subsequent transphosphorylation of their intracellular domains. With no known ligand, HER2 is assumed to play a regulatory role

through its heterodimerization with other HER family members or homodimerization with itself when expressed at very high levels [138]. Additionally, HER2 can also heterodimerize with other membrane receptors, for example with insulin-like growth factor receptor I [139]. Furthermore, HER2 has the strongest catalytic kinase activity and, as such, HER2 heterodimers have the strongest signaling activity. The phosphorylated tyrosine residues activate several downstream intracellular signaling molecules, including the PI3K/AKT, RAS/MEK/MAPK and STAT kinase cascades, ultimately regulating transcription factors of genes involved in cell proliferation, survival, differentiation, angiogenesis, invasion and metastasis (Figure 7) [136].

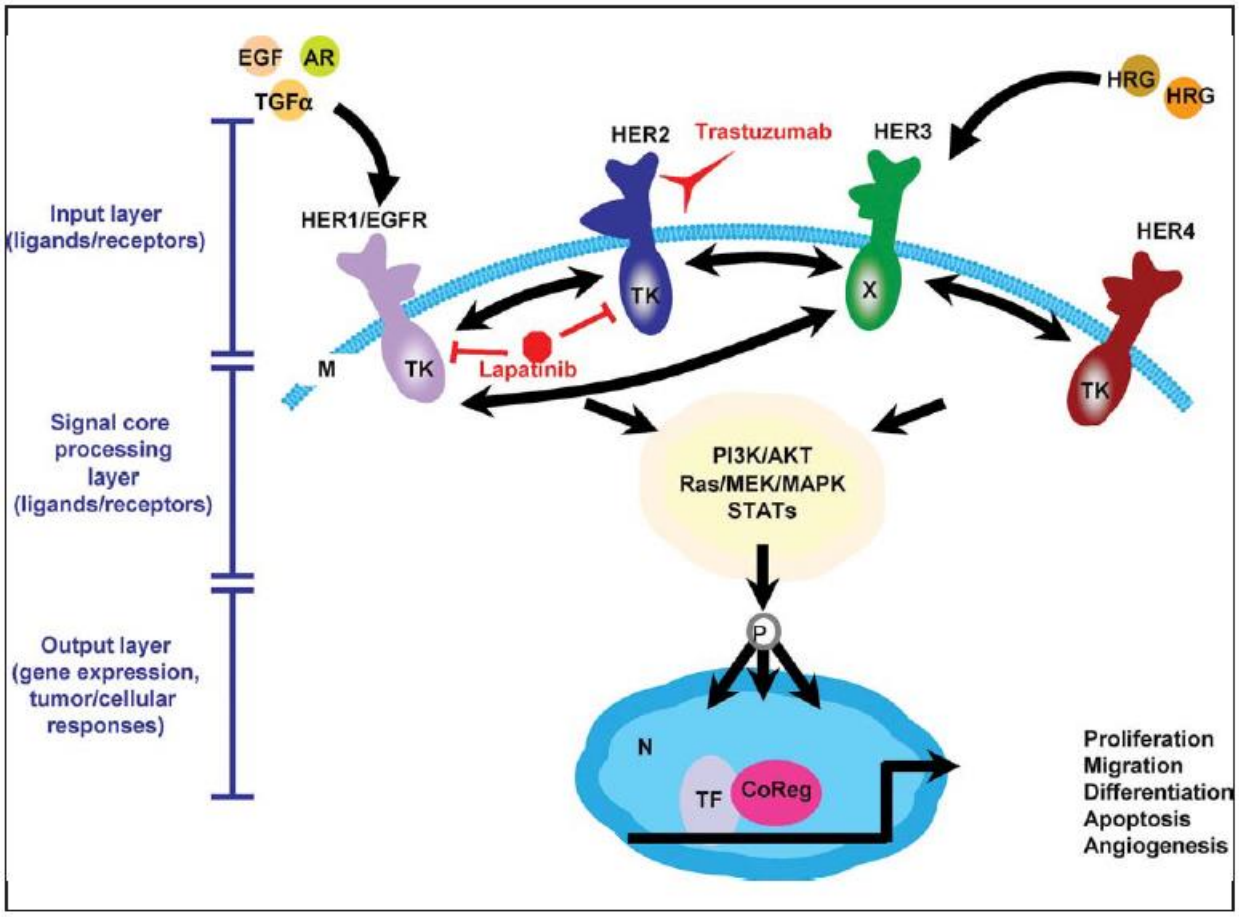


Figure 7

The HER signaling network and HER2-targeted therapy in breast cancer (from Gutierrez and Schiff [136])

The HER2 gene is located on the long arm of chromosome 17 (17q12) and was found to be amplified in human BC cell line more than 30 years ago, and shortly proved to be highly relevant in the pathogenesis and progression of BC [140,141]. Additionally, numerous studies have shown that HER2 gene amplification can already be present in CIS of the breast, the immediate precursor of invasive carcinoma, and that frequently HER2 *status* is concordant with the invasive component [142,143].

In invasive BC, HER2 is amplified and/or overexpressed in approximately 15-20% of the cases, representing a clinically important subset of BC associated with higher metastatic potential and poor clinical outcome but also with a high likelihood of response to HER2-targeted therapy [104,141,144-148]. HER2-positve BCs are correlated with particular pathological characteristics, such as high histologic grade, low ER and PgR expression, and high proliferation rates. Clinically, these BCs show a tendency to metastasize to the central nervous system and to visceral organs, as well as present an increased sensitivity to doxorubicin and resistance to endocrine therapy [136]. The increased sensitivity to doxorubicin is presumably explained by the co-amplification of topoisomerase-2 gene (TOP2A), which is located near the HER2 locus [149].

Importantly, the poor patient prognosis in HER2-positive BC is deeply overcome with HER2 targeting therapy. Available therapeutic drugs for HER2-positive BC include humanized monoclonal antibodies (trastuzumab and pertuzumab), tyrosine kinase inhibitors (lapatinib) and trastuzumab-emtansine (T-DM1), an antibody-drug conjugate [137]. Trastuzumab, the first antibody targeting HER2, is well tolerated by patients, with little toxicity, which is due to its specificity for HER2 overexpressing cancer cells [136]. Although the mechanism of action of monoclonal antibodies are not fully understood, they recognize the external domain of HER2, inhibiting downstream signaling [150]. In contrast, lapatinib is a tyrosine kinase inhibitor,

representing a drug with a different mechanism of action against HER2 protein. This drug is still effective in BC patients with the truncated variant of HER2 protein (named p95), which is resistant to trastuzumab and pertuzumab [136]. This aberrant form, without the extracellular domain, is constitutively active due to the absence of inhibitory signals from the external domain. As such, in this setting, antibodies that target the external domain of HER2 (either for targeted therapy or for IHC analysis) are useless [151].

HER2 targeted therapy appears to benefit mainly tumors overexpressing HER2 protein or with HER2 gene amplification, making the accuracy of HER2 testing an important practical problem for successful treatment. At present, HER2 evaluation is most frequently performed by IHC, which detects HER2 protein overexpression, resulting in three possible outcomes: negative (score of 0 or 1+), equivocal (score of 2+) and positive (score of 3+). In cases of equivocal results, reflex testing should be performed on the same specimen with ISH assays for the assessment of HER2 gene amplification (Figure 8) [97].

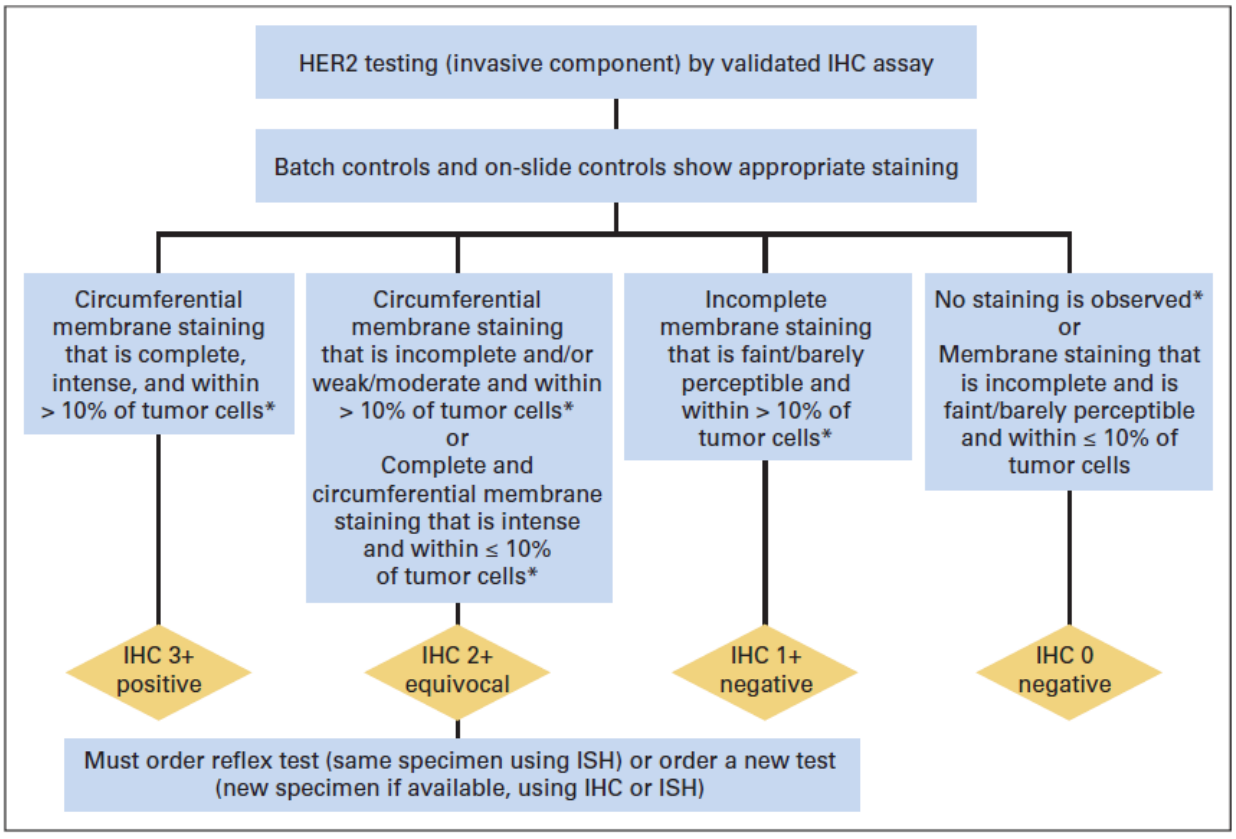


Figure 8

Algorithm for evaluation of HER2 protein expression by IHC assay (from Wolff et al [97])

The principles of ISH assay rely on labeled DNA probes that hybridize to genomic sequences of interest through base pair complementarity. Fortunately, this method can be applied on several types of samples, from cell lines to frozen or paraffin-embedded tissues [152]. Fluorescence ISH (FISH), the more traditional assay, requires a fluorescence microscope with multiband filters and specific training. Manual counting is time consuming and automated counting requires a high-resolution digital camera [136]. Bright-field ISH, unlike FISH, presents some advantages, such as the use of a light microscope, permanent staining and easy identification of target cells, which can detect tumor heterogeneity more easily [153]. Manual counting is less time consuming and more easily performed by pathologists given the resemblances with IHC [154].

The new 2013 ASCO/CAP guideline has updated the definition of HER2-positive *status* by modifying both IHC and ISH criteria, reducing the thresholds for post-analytical interpretation of positive results in comparison with the previous 2007 ASCO/CAP guideline [97,155]. In the new guideline, a HER2 score 3+ is defined as the presence of complete and intense membrane staining, in at least 10% of tumor cells [97]. This represented a return to the IHC criteria originally used in the first-generation clinical trials [156]. A similar approach was used regarding ISH criteria. In the updated guideline, a case is considered HER2-positive if HER2/CEP17 ratio is higher than 2.0, as initially used. Moreover, it was added that, even in assays with internal control probes, a case is also considered HER2-positive if the average of HER2 copy number per cell is higher than 6.0, regardless of the value of HER2/CEP17 ratio (Figure 9).

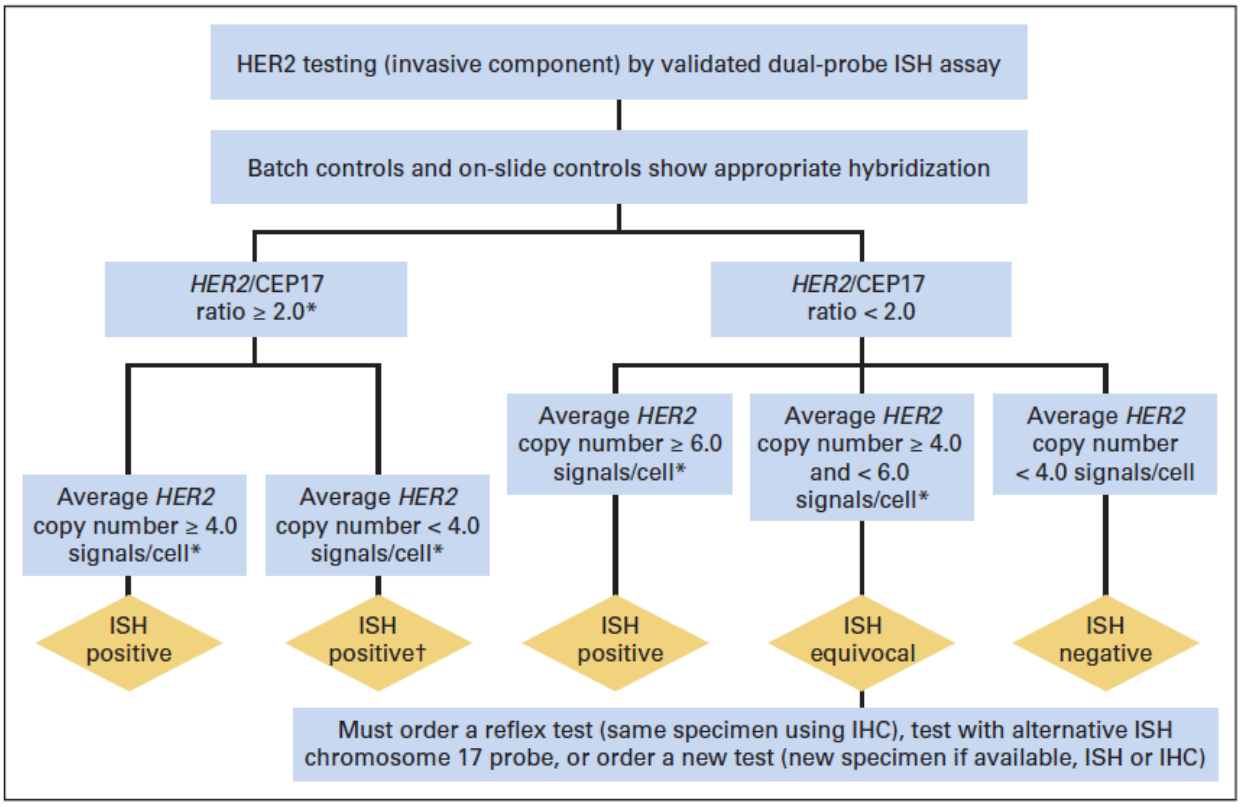


Figure 9

Algorithm for evaluation of HER2 gene amplification by ISH assay (from Wolff et al [97])

Regarding ISH assays, the 2013 ASCO/CAP guideline accept bright-field ISH and recommend counting at least 20 non-overlapping cells in two separate areas of invasive cancer [97]. Although this is usually interpreted as counting a total of 40 cells (at least 20 cells per area), the supplementary data of the guidelines actually explain that the minimum cell number is, in fact, a total of 20 cells in two separate areas of invasive cancer (at least 10 cells per area). It has already been shown that high ISH interobserver reproducibility exists; nevertheless, the minimum number of cells that should be counted to obtain a reproducible result is yet to be determined [157,158]. Even if the current ASCO/CAP guideline recommendations are adhered to, the imprecision of HER2 testing remains a relevant issue, for both IHC and ISH techniques [159,160].

Heterogeneity has been notice in almost all types of cancer, including BC, being related to several aspect of disease progression and clinical outcome [161]. The first recommendation regarding HER2 genetic heterogeneity (HER2-GH) was published in 2009 as an extension of the 2007 ASCO/CAP HER2 guidelines after the acknowledgment that some tumors displayed intratumoral heterogeneity and such cases could originate discrepant results between IHC and ISH analysis [162]. At that time, HER2-GH was defined as HER2 gene amplification in 5 to 50% of invasive cancer cells. Importantly, the definition was based on studies that did not include clinical outcome, being the first step to investigate the clinical significance of HER2-GH and the possible role of target-therapy in this setting [163,164]. Thereafter, numerous studies have shown that HER2-GH could be present in BC from 5% to 40% of the cases [165,166]. Additionally, it was shown that HER2-GH was more frequent in cases near the threshold of positivity and that heterogeneity measured in individual cells is not informative of clonal heterogeneity within a tumor population [167].

Currently, the definition of HER2-GH has changed from individual cells to discrete population of tumor cells with HER2 gene amplification. According to the 2013 ASCO/CAP HER2 guideline, a tumor is considered HER2 positive if HER2 gene amplification is present in at least 10% of the total tumor cell population [97].

Genomic heterogeneity has important practical implications. It has been documented that some tumors lose HER2 expression after treatment with trastuzumab, probably through a positive selection of a HER2-negative clone not sensible to anti-HER2 targeted therapy. Likewise, some tumors can gain HER2 expression over time, especially after endocrine therapy targeting ER, representing either a selection of a HER2-positive clone in an initial heterogeneous tumor or a new genetic acquisition in an original HER2-negative tumor [136].

1.3 Tumor infiltrating lymphocytes (TILs)

Cancer development and progression is dependent on a complex system of different factors, including genetic and epigenetic alterations, and on factors from the tumor microenvironment, such as stromal and immune cells.[10] In fact, in recent years, numerous studies have focused on the presence and function of the host immune system and its relationship with tumor progression in a variety of solid tumors, including BC, showing that spontaneous intratumoral lymphocytic infiltrate is related to patient prognosis.[168-174]

In colorectal cancer, the presence of peritumoral lymphocytic infiltrate has been correlated with tumors with microsatellite instability (MSI), a known factor associated with better prognosis [175]. Additionally, the presence of effector memory T cells was related with tumors without pathological signs of early metastatic invasion, supporting an active anti-tumor immune response. Moreover, an elevated number of the same cells were independently correlated with an improved clinical outcome [168]. Similarly, in different models such as melanoma, intratumoral lymphocytic infiltrate was associated with histological regression and absence of regional lymph node involvement [176]. Lastly, in squamous cell carcinoma of the head and neck (SCCHN), the presence of functional T cells is also associated with better clinical prognosis [174].

Stromal TILs include several types of T and B lymphoid cells, along with macrophages, natural killer (NK) cells and dendritic cells, in different proportions with distinct associations with clinical outcome. For instance, CD4+ T helper 1 (Th1) cells, CD8+ cytotoxic T cells, NK cell, M1 macrophages and dendritic cells have a protective role against tumor growth. Inversely, CD4+/FOXP3+ immunosuppressive cells, CD4+ Th2 cells and M2 macrophages promote tumor growth [177]. In fact, cytotoxic T cells are significantly increased in high grade, ER-negative BC

and BC with increased proliferative activity, as well as associated with improved clinical outcome.[178-180] On the other side, immunosuppressive T cells have been shown to be associated with worse prognosis in invasive BC and even with increased risk of relapse in ductal CIS (DCIS).[181-183] This data suggest that cytotoxic T cells are responsible for the antitumor immune activity and immunosuppressive T cells can inhibit this response. Furthermore, B lymphocytes are also associated with higher histological grade, ER-negative cases and basal phenotype, as well as with better prognosis.[184] In the same way, T follicular helper (Tfh) cells are important to develop and attract memory B cells, being associated with increase response to conventional chemotherapy in BC [177]. This indicates that humoral immune response, along with cell-mediated immune response, acts in convergence to achieve effective anti-tumor response. Based on this knowledge, it would be important to evaluate the clinical value of subtyping the composition of stromal TILs in BC and reveal the potential predictive role of these markers in response to immunotherapy. Nonetheless, it is remarkable that the quantification of stromal TILs alone, regardless of its specific subpopulation of lymphoid cells, has prognostic and predictive information. Curiously, there is no cut-off regarding the evaluation of stromal TILs, given that its prognostic value follows a continuous scale: each 1% increment is associated with an increase in the level of pathologic complete response (pCR) [177].

Although the BC's inflammatory infiltrate has been studied for several decades with conflicting results, large cohorts have recently shown an association between the presence of stromal tumor-infiltrating lymphocytes (TILs) with improved prognosis and better response to neoadjuvant chemotherapy, regardless of the absence of information of its specific immune cells.[185,186] The association between stromal TILs at diagnosis with disease-free and overall survival has been shown to be significant only in TNBC and HER2-positive BC [177]. In contrast,

luminal BCs, apparently, are less immunogenic probably due to the low mutation rate when compared to HER2-positive and TNBCs [177].

Tumor cell death by conventional therapies, like chemotherapy and radiotherapy, causes the release of tumor-associated antigens, which in turn are used by antigen presenting cells (APCs) to stimulate downstream effector cell and increase anti-tumor response. In TNBC, for instances, the presence of stromal TILs in tumor tissue at diagnosis associates with better patient outcome after adjuvant anthracycline-based chemotherapy.[187] Likewise, in HER2-positive BC, the number of TILs in tumor tissue associates with a better response to trastuzumab treatment.[188] In the same way, radiotherapy was shown to increase the variability of the T-cell receptor in intratumoral T cells [189].

Additionally, the relationship between stromal TILs in BC and the response to neoadjuvant chemotherapy (NAC) has also been already evaluated. Several studies demonstrated higher stromal TILs as an independent marker for pCR after NAC [178,185,186,188,190]. In addition, the expression of inflammatory genes was also associated with stromal TILs and pCR, specifically T cell markers [185,191]. Furthermore, NAC significantly decreases immunosuppressive T cells on surgical specimens, while the number of cytotoxic T cells remains constant, being associated with pCR [192]. As such, an increase in stromal TILs in the neoplastic tissue after NAC is obviously associated with improved prognosis, as well as the ratio of cytotoxic T cells to immunosuppressive T cells [177]. This means that chemotherapy not only acts through the direct action against tumor cells, but also through the induction of an antitumor immune response, by causing a differential change in the composition of stromal TILs, specifically decreasing immunosuppressive T cells. Moreover, the use of immunotherapies that decrease de number of

immunosuppressive cells might add an increase in the efficacy of neoadjuvant and adjuvant chemotherapy.

1.4 Programmed cell death-ligand 1 (PDL1) expression

Tumor cells express new antigens that should be recognized by patient's immune system, although most of the time the immunologic response is unable to eliminate cancer cells. Currently, many efforts have been made to identify molecular mechanisms that enable tumor cells to escape from the host immune system.[10] An example of such immune checkpoint pathway is the expression of programmed cell death-ligand 1 (PDL1) by neoplastic cells conveying an inhibitory signal to T lymphocytes through the interaction with its receptor programmed cell death protein 1 (PD1). The PD1/PDL1 signaling pathway is present in physiologic processes to control self-tolerance and self-damage by normal tissues, being manipulated by tumor cells to protect from elimination by the immune system (Figure 10) [193].

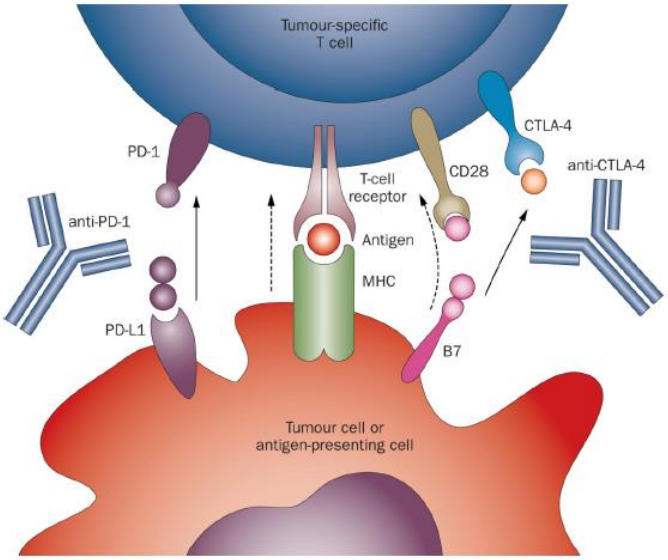


Figure 10

Immune checkpoint blockade (from Drake et al [194])

PD1 is a cell surface protein that is part of the CD28 family, being expressed mainly on activated T-cells. Nevertheless, PD1 is also present on other activated lymphoid cells, such as B cells, NK cells and monocytes, which means that regulates T-cell independent immune surveillance [195]. PD1 is encoded by the PDCD1 gene, located in chromosome 2q37, and has two main ligands: programmed cell death-ligand 1 (PDL1) and 2 (PDL2), both located in chromosome 9p24.1 (PDCD1LG1 and PDCD1LG2, respectively) [193].

The specific binding of either PDL1 or PDL2 to PD1 leads to the activation of inhibitory kinases responsible for T-cell proliferation as well as cytokine production and secretion.[196-199] A few differences between the expression of PDL1 and PDL2 need to be referred. PDL1 is mainly expressed on immune cells (for instance, T cells, B cells, macrophages and dendritic cells) along with non-immune cells, such as vascular endothelial cells and, obviously, tumor cells. PDL2 is mostly limited to APCs, such as macrophages and dendritic cells [193].

PDL1 is expressed in various solid tumors and hematopoietic tumors, being currently evaluated in non-small cell lung carcinoma (NSCLC) and urothelial carcinoma, since it brings additional information to patient prognosis and to the selection of immunotherapy currently available.[200] On the other way, PDL2 is mostly expressed in hematologic malignancies [193].

Several signaling pathways have been proved to regulate PDL1 expression in various cancer models. There are two main pathways regulating the escape of tumor cells from immune surveillance. The first pathway comprises an innate/intrinsic immune response triggered by active oncogenic signaling, such as the PTEN/PI3K/AKT or ALK/STAT3 pathways, that are known to up-regulate PDL1 expression in glioblastomas and ALK-positive T-cell lymphoma and NSCLC, respectively [201-203]. The second pathway represents an adaptive immune response

induced by inflammatory signals, such as interferon gamma and interleukins, that have been shown to up-regulate PDL1 expression on TILs and tumor cells [204].

A classification system into 4 classes has been proposed combining the presence or absence of stromal TILs with the expression of PDL1 [205]. Therefore, type I represents tumors with stromal TILs and PDL1 expression (adaptive immune resistance), type II represents tumors without stromal TILs and no PDL1 expression (immune ignorance), type III represents tumors without stromal TILs but with PDL1 expression (intrinsic induction) and type IV represents tumors with stromal TILs but no PDL1 expression (immune tolerance by other suppressors).

Type I and type IV tumors are probably the ones most likely to respond to immune checkpoint inhibitors, leaving stromal TILs in the tumor microenvironment to act against tumor cells. Although type I tumors would benefit from PD1/PDL1 targeted-therapy, type IV tumors, which do not have PDL1 expression, would probably profit from targeting other immune checkpoint inhibitors. On the other side, in type II and III tumors, which do not have preexisting stromal TILs, make therapies planned to recruit T cells to the tumor site, such as vaccination or even conventional chemotherapy/radiotherapy that promotes immunogenic action against tumor cells, probably useful approaches. Furthermore, type III tumors, with innate/intrinsic PDL1 induction through active oncogenic signaling, would also benefit from adding PD1/PDL1 targeted-therapy to induce a more effective anti-tumor immune response.

Although contradictory information has been published regarding PDL1 expression and patient prognosis, in theory, the expression of PDL1 in the neoplastic tissue should have an adverse effect on prognosis, given the inhibitory effect of PDL1 in the antitumor immune response.[206] However, it has been reported that PDL1 expression can be related with better

survival rates in several tumor models, probably due to the close association of PDL1 with the presence of increased stromal TILs, the effectors against tumor cells.[207,208]

The use of specific monoclonal antibodies targeting either PD1 or PDL1 has been shown to promote tumor cell death induced by the host immune system in many cancer models.[198,209] For instance, nivolumab, the first FDA-approved humanized monoclonal antibody against PD1, has been selected for several solid tumors, including melanoma, NSCLC, renal cell carcinoma (RCC), classical Hodgkin lymphoma, SCCHN, urothelial carcinoma and even colorectal carcinoma with mismatch repair deficiency (dMMR) or MSI. Pembrolizumab, an alternative anti-PD1 antibody, has also been approved for melanoma, NSCLC, SCCHN, classical Hodgkin lymphoma and urothelial carcinoma, as well as any solid tumor with dMMR or MSI. Regarding anti-PDL1-targeted therapy, atezolizumab has been approved for urothelial carcinoma and NSCLC, and avelumab for Merkle cell carcinoma and urothelial carcinoma [193].

There are a few characteristics to be mentioned regarding the inhibition of PD1 or PDL1. The inhibition of PD1 prevents the interaction of both ligands (PDL1 and PDL2), which can increase the probability of toxicity, and does not block the ligation of PDL1 to the costimulatory molecule CD80, leaving PDL1 still partially active. On the contrary, the inhibition of PDL1 blocks the ligation of both PD1 and CD80, leaving intact the interaction of PDL2 to PD1, which can preserve immune tolerance and explain the fewer immune-related side effects in patients treated with anti-PDL1 [205].

The combination of PD1 and PDL1 inhibitors with conventional chemotherapy as well as with other targeted-therapy (such as EGFR, ALK or BRAF) has been shown to be improve patient outcome in various clinical trials [193]. This means that cancer immunotherapy, by modulating the immune system to increase its response towards tumor cells, adds an additional

effect to current therapy, representing a valuable new strategic tool to be used in the treatment of cancer. Evidently, all drugs have unwanted actions and both anti-PD1 and anti-PDL1 options have an increased risk of developing auto-immune side effects. The most affected organs with immune-related manifestations are the skin, the gastrointestinal system, the liver and the lungs [193].

Given the potential toxicity that PD1/PDL1 targeted therapy can present, it would be useful to have a predictive biomarker that helped in the selection of patients who most likely benefit from these treatments. The expression of PDL1 by IHC has been explored as a possible predictive biomarker with some conflicting results. For instance, nivolumab was shown to achieve objective responses in about a third of patients with PDL1-positive tumors (melanoma, NSCLC and RCC) and no response in patients with PDL1-negative tumors [210]. In the same way, pembrolizumab was also shown to reach significantly higher response rates in patients with PDL1-positive NSCLC [211]. Similarly, atezolizumab was also shown to accomplish better results in several cancer patients with PDL1-positive tumors, such as RCC and urothelial carcinoma [212,213].

Interestingly, it has also been shown that objective responses to nivolumab can still occur in patients with PDL1-negative squamous cell carcinoma of the lung [214]. In fact, a recent meta-analysis showed clinical responses significantly different in about a third of patients with PDL1-positive tumors and in about a fifth of patients with PDL1-negative tumors when treated with nivolumab, pembrolizumab or atezolizumab. Additionally, the predictive value of PDL1 expression was even greater in NSCLC and melanoma patients as well as in nivolumab and pembrolizumab treated patients [215].

Although some inconsistencies exist, there is enough evidence for a link between PDL1 expression and efficacy of PD1/PDL1 targeted-therapy. The different results of PDL1 expression are probably related to different methods in evaluating PDL1 expression, different thresholds values and monoclonal antibodies used as well as heterogeneity within tumors.[216] Currently, the assays to evaluate the role of PDL1 expression for prognosis and predictive information are not standardized neither in methodology, nor interpretation of the staining. In practice, there are several companion diagnostic tests measuring the expression of PDL1, in both tumor cells and stromal TILs, using different monoclonal antibodies associated with specific drugs. Therefore, nivolumab, pembrolizumab and atezolizumab are associated with clone 28-8 from Dako, clone 22C3 from Dako and clone SP142 from Roche Ventana, respectively [217].

Recently, a working group named Blueprint Project was created by an industrial-academic collaborative partnership, including medical societies, pharmaceutical and diagnostic companies, as well as regulatory agencies. The objective was to evaluate the expression of 4 different PDL1 assays in NSCLC, assessing the possibility of interchangeable results. Initial results show that clones 22C3 and 28-8 from Dako have comparable precision in the evaluation of PDL1 expression in tumor cells whereas SP142 from Roche Ventana systematically stains fewer tumor cells. Additionally, it was also demonstrated that there is higher variability in the evaluation of PDL1 expression in immune cells than in tumor cells [218]. In conclusion, almost 40% of the cases could present discordant classification making the use of different antibodies to predict targeted therapy other than the already approved very problematic.

CHAPTER 2

THESIS GOAL AND SPECIFIC AIMS

The overall goal of this thesis was to improve the value of prognostic and predictive biomarkers in breast cancer pathology. With this purpose, we focused the study on specific biomarkers:

1) the oncogenic receptor tyrosine kinase HER2, which expression is already routinely assessed in the clinical practice due to its sustained prognostic and predictive value;

2) the presence of stromal TILs and the expression of PDL1 to evaluate the possibility of using immunotherapy to treat BC patients in the near future.

Regarding the oncogene HER2, which overexpression and gene amplification is evaluated in all BC cases to identify patients that will most probably benefit of being treated with HER2 targeted therapy, our specific aims were the following:

1 - To evaluate the impact of the recent alterations introduced on the ASCO/CAP guidelines on the result of HER2 gene amplification test, by the use of bright-field ISH (Chapter 3);

2 - To evaluate the intraobserver and interobserver interpretative reproducibility of the HER2 gene amplification assay, by measuring the impact of counting increasing numbers of invasive cancer cells (Chapter 4);

3 - To evaluate the heterogeneity of HER2 gene amplification in invasive and in in situ BC cases (Chapter 5).

Regarding stromal TILs and PDL1 expression, the specific aim was to assess the clinical impact of the expression of these biomarkers in BC, namely their correlation with classical pathological features, cancer molecular subtypes, as well as patients' prognosis (Chapter 6).

CHAPTER 3

APPLICATION OF THE 2013 ASCO/CAP GUIDELINE AND THE SISH TECHNIQUE FOR HER2 TESTING OF BREAST CANCER SELECTS MORE PATIENTS FOR ANTI-HER2 TREATMENT

(Polonia A et al. *Virchows Arch.* 2016;468(4):417-423)

3.1 Abstract

The aim of this study is to assess the impact of changes of the 2013 ASCO/CAP guideline on the results of HER2 testing in breast cancer. A series of 916 primary invasive breast cancer cases, assessed as HER2 2+ by IHC in part using the 2007 and in part the 2013 ASCO/CAP criteria, was evaluated for HER2 amplification *status* by SISH and classified according to both 2007 and 2013 ASCO/CAP ISH guideline criteria. We observed a significant increase of HER2-positive cases (12.4% to 16.8%) and a decrease of HER2-equivocal cases (3.6% to 0.7%). Of the cases studied, 52.1% fulfilled both criteria of HER2/CEP17 ratio and average HER2 copy number *per* cell to be classified as HER2-positive. Reclassification of the cases from before the introduction of the new ASCO/CAP guideline with the 2013 ISH criteria resulted in an increase of cases with a HER2-positive status (12.4% to 14.2%) and in a decrease of HER2-equivocal cases (3.6% to 1.6%). The 2013 ASCO/CAP guideline selects more patients for anti-HER2 targeted therapy, mostly based on the modifications of criteria to evaluate ISH-HER2.

3.2 Introduction

In the western world, breast cancer (BC) is the most commonly diagnosed malignancy among women, representing about 30% of all new cancer cases, and after lung cancer the second leading cause of cancer death [219,220]. The current cancer care guidelines for BC recommend that estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor 2 (HER2) status must be routinely determined in all patients with invasive BC, BC recurrence and BC metastases [75,97]. These guidelines were published to help improve laboratory performance in the determination of these markers, which provide useful predictive information regarding response to targeted therapy.

HER2, located on the long arm of chromosome 17 (17q12), is amplified and/or overexpressed in about 15% to 20% of invasive BC. Cases with a HER2-positive status represent a clinically important subset of BC associated with poor outcome but also with a high likelihood of response to HER2-targeted therapy [104,141,147,148]. Several studies have shown that anti-HER2 therapy given during and/or after chemotherapy results in a significant improvement in disease-free and overall survival [103,105,106]. Therefore, HER2 is a helpful marker for therapy decision making in patients with BC and appropriate evaluation of HER2 status ensures that the right patient receives the right treatment [97].

At present, HER2 protein expression is determined in BC samples by immunohistochemistry (IHC) resulting in three possible outcomes: negative (score 0 or 1+), equivocal (score 2+), and positive (score 3+). If the IHC result is equivocal, reflex testing should be performed on the same specimen using an alternative assay, such as in situ hybridization (ISH) [97].

The new 2013 ASCO/CAP (American Society of Clinical Oncology/College of American Pathologists) guideline has updated the definition of HER2-positive status by modifying both IHC and ISH criteria, reducing the thresholds for the post-analytical interpretation of positive results in comparison with the previous 2007 ASCO/CAP guideline [97,155]. In the new guideline, a HER2 score 3+ is defined as the presence of complete and intense membrane staining, in at least 10% of tumor cells [97]. This represented a return to the IHC criteria originally used in the first-generation clinical trials [156]. A similar approach was used regarding ISH criteria (see below).

In this study, we aim to compare the impact of the change from the 2007 to the 2013 ASCO/CAP guidelines on the result of HER2 amplification test in BC.

3.3 Materials and methods

Cases

A series of 916 primary invasive BC cases was retrieved from the archives of Ipatimup Diagnostics, including cases evaluated one year before (494 cases from November 2012 to October 2013) and one year after (422 cases from December 2013 to November 2014) the publication of the new ASCO/CAP guideline (November 2013). All BC cases (core biopsies and surgical specimens) had been fixed in 10% formalin, embedded in paraffin, and were referred to our institution (national reference center for HER2 ISH) with an equivocal IHC HER2 score (2+) to perform the HER2 amplification assay with a silver marker (SISH).

Ethics approval and informed consent were not required for this study.

SISH

SISH testing was performed on 3-µm sections of formalin-fixed, paraffin-embedded tissue of all BC cases using dual-hapten, dual-colour ISH. The dual-probe assay (INFORM HER2 Dual ISH DNA Probe Cocktail Assay; Ventana Medical Systems, Inc., Tucson, Arizona) contains a HER2 locus-specific probe and a control probe specific for the centromere of chromosome 17 (CEP17). The entire procedure was carried out on an automated staining system (Ventana BenchMark^{TM} XT Staining System) according to the manufacturer's instructions. Positive and negative controls were used for each staining run.

Evaluation of the results included recording the number of HER2 and CEP17 signals in at least 20 nuclei in two different areas. The samples were classified by pathologists (AP and FS) according to the 2007 and 2013 ISH criteria for HER2 amplification. Corresponding

hematoxylin and eosin staining were used for the identification of the invasive component of the tumor.

The 2007 ASCO/CAP guideline defines HER2 amplification as positive at a HER2/CEP17 ratio >2.2, equivocal at a HER2/CEP17 ratio ≤2.2 and ≥1.8, and negative at a HER2/CEP17 ratio <1.8 [155]. The 2013 ASCO/CAP guideline establishes the result of HER2 amplification as positive at a HER2/CEP17 ratio ≥2.0 or a HER2/CEP17 ratio <2.0 and an average HER2 copy number per cell of ≥6.0, equivocal when HER2/CEP17 ratio <2.0 and average of HER2 copy number ≥4.0 and <6.0 signals per cell, and negative when HER2/CEP17 ratio <2.0 and average HER2 copy number of <4.0 signals per cell [97].

Chromosome 17 polysomy was defined as an average of ≥ 3.0 CEP17 signals per cell [166]. Genomic heterogeneity was also recorded and considered present if a discrete population of tumor cells with HER2 amplification represented at least 10% of the total tumor cell population [97].

Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 21.0 for Windows. The Pearson´s chi-squared (χ^2) test and McNemar test were used for comparison of qualitative variables and the *t* test for quantitative variables. The level of significance was set at $p < 0.05$.

Agreement rates between the 2007 and the 2013 ASCO/CAP guidelines regarding HER2 gene amplification assay were evaluated with kappa (*k*) statistics [221].

3.4 Results

The 916 BC cases concerned 97.2% women and 1.2% men. The age ranged from 24 to 103 years, with a median age at diagnosis of 59 years.

The distribution of gender, age, HER2/CEP17 ratio, and average HER2 copy number per cell were not statistically different between the pre- and post-new guideline cases (Table 1 and Fig. 1). The only parameters that changed significantly with the new guideline were the average CEP17 copy number per cell (1.98 to 1.78; $p<0.001$; Table 1) and the presence of chromosome 17 polysomy (4.1% to 0.9%; $p=0.003$; Table 1).

Table 1 Differences between the cases before and after the introduction of the 2013 ASCO/CAP guideline

	Cases before 2013 ASCO/CAP guideline	Cases after 2013ASCO/CAP guideline	*p*
Gender (female / male / NI)	481 / 6 / 7	409 / 5 / 8	ns (0.974)[a]
Age (mean ± sd)	58.17 ± 13.76	59.12 ± 14.10	ns (0.346)[b]
HER2/CEP17 ratio (mean ± sd)	1.68 ± 1.57	1.68 ± 1.50	ns (0.930)[b]
Average of HER2 copy number per cell (mean ± sd)	3.17 ± 2.56	2.88 ± 2.42	ns (0.077)[b]
Average of CEP17 copy number per cell (mean ± sd)	1.98 ± 0.51	1.78 ± 0.42	**<0.001**[b]
Chromosome 17 polysomy (present / absent)	20 (4.1%) / 474 (95.9%)	4 (0.9%) / 418 (99.1%)	**0.003**[a]

NI – not informed; sd – standard deviation; ns – not significant; [a] – Pearson´s chi-squared test; [b] – *t* test

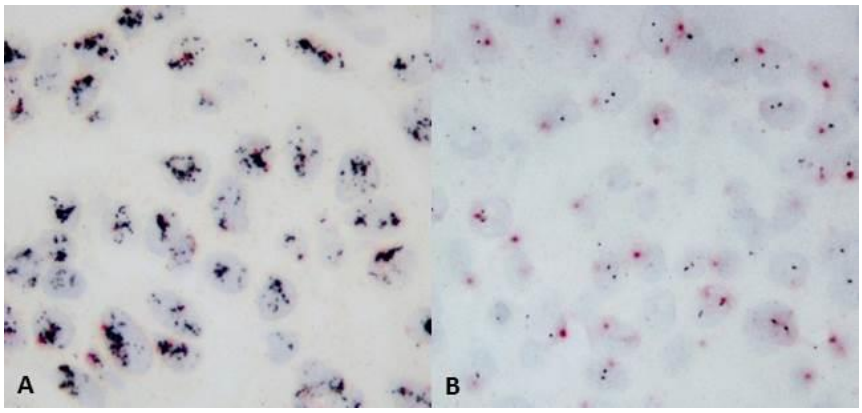


Figure 1 Examples of results of HER2 detection by SISH technique (x400)
A: HER2-positive / B: HER2-negative

Table 2 and Fig. 2A present the results of HER2 test performed on the pre-new guideline cases (using the ISH criteria from the 2007 ASCO/CAP guideline): 415 cases (84.0%) HER2-negative, 18 cases (3.6%) HER2-equivocal, and 61 cases (12.4%) HER2-positive. Table 2 and Fig. 2B present the results of HER2 test performed on the post-new guideline cases (using the ISH criteria from the 2013 ASCO/CAP guideline): 348 cases (82.5%) HER2-negative, 3 cases (0.7%) HER2-equivocal, and 71 cases (16.8%) HER2-positive. The differences are statistically significant (Table 2 – statistical analysis A; p=0.003). We also observed that 52.1% of the positive cases (37/71) fulfill both criteria of HER2/CEP17 ratio ≥2.0 and average of HER2 copy number per cell ≥6.0 (Table 3 and Figure 2B). We furthermore classified the pre- and post-new guideline cases using the 2007 and 2013 ISH criteria and observed a slight but non-significant increase in HER2-positive cases and a similar decrease in HER2-equivocal cases (Table 2 – statistical analysis B and C; p=0.185 and p=0.261, respectively).

Table 2 Classification of HER2 test according to the 2007 and 2013 ISH criteria

HER2 result	Cases before 2013ASCO/CAP guideline		Cases after 2013ASCO/CAP guideline	
	ISH criteria 2007	ISH criteria 2013	ISH criteria 2007	ISH criteria 2013
Positive	12.4% (61)	14.2% (70)	15.9% (67)	16.8% (71)
Equivocal	3.6% (18)	1.6% (8)	2.4% (10)	0.7% (3)
Negative	84.0% (415)	84.2% (416)	81.7% (345)	82.5% (348)
Total	494		422	
Statistical analysis	A			A
	B		B	
		C		C
	D	D	E	E

A - Cases before (ISH criteria 2007) vs after (ISH criteria 2013) 2013 ASCO/CAP guideline: p=**0.003**[a]

B - Cases before vs after 2013ASCO/CAP guideline (ISH criteria 2007): p= 0.185[a]

C - Cases before vs after 2013ASCO/CAP guideline (ISH criteria 2013): p= 0.261[a]

D - Cases before 2013ASCO/CAP guideline – ISH criteria 2007 vs ISH criteria 2013: p=**0.011**[b]

E - Cases after 2013ASCO/CAP guideline – ISH criteria 2007 vs ISH criteria 2013: p=0.071[b]

[a] – Pearson´s chi-squared test; [b] – McNemar test

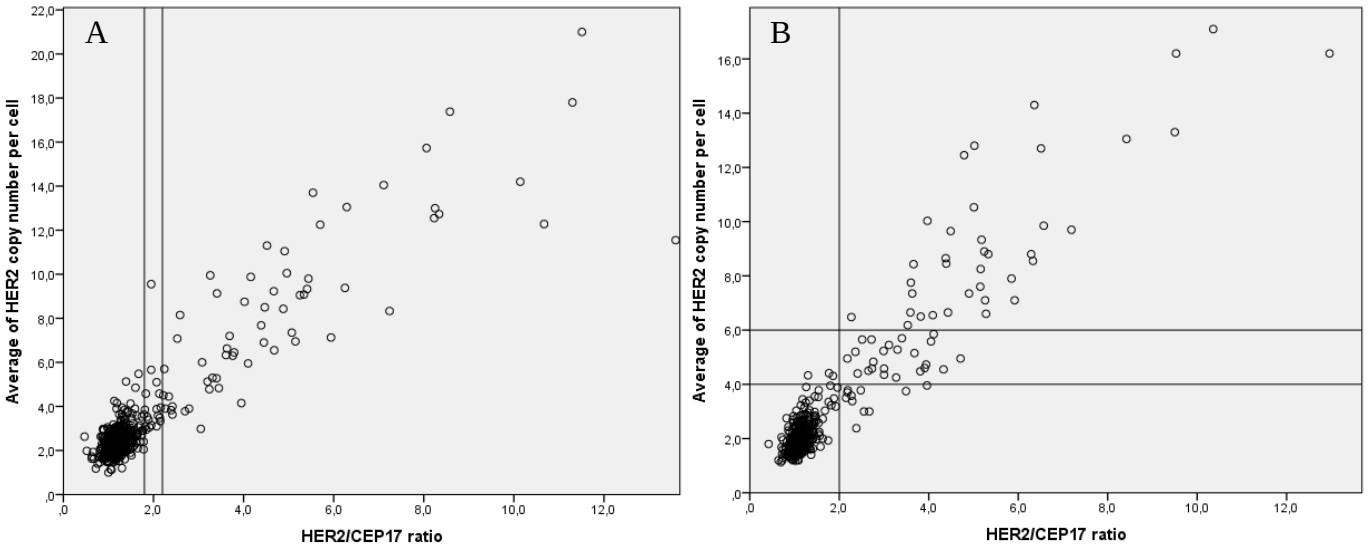


Figure 2 Cases before (A) and after (B) the introduction of the 2013 ASCO/CAP guideline

Table 3 Classification of the cases after the 2013 ASCO/CAP guideline

HER2/CEP17 ratio	**Average of HER2 copy number signals per cell**		
	<4.0	≥4.0 and <6.0	≥6.0
< 2.0	348	3	0
≥ 2.0	11	23	**37**

In the reclassification of the two case series using the 2007 and 2013 ISH criteria, we observed an increase in HER2-positive cases (12.4% to 14.2% and 15.9% to 16.8%, respectively) and a decrease in HER2-equivocal cases (3.6% to 1.6% and 2.4% to 0.7%, respectively). This was statistically significant in the pre-new guideline cases (Table 2 – statistical analysis D; p=0.011) and near significant in the post-new guideline cases (Table 2 – statistical analysis E; p=0.071).

In the pre-new guideline cases, the 2013 ISH criteria reclassified 22 (4.5%) of the cases, 9 as HER2-positive (from HER2-equivocal), 7 as HER2-negative (from HER2-equivocal), and 6 as HER2-equivocal (from HER2-negative). All HER2-positive cases according to the 2007 guideline remained HER2 positive with the 2013 guideline. Therefore, the concordance rate between 2007 and 2013 ISH criteria was 95.5% (472/494), with an agreement rate of 0.838 (p<0.001) (Table 4).

Table 4 Classification of the cases before the 2013 ASCO/CAP guideline

2007 ISH criteria	2013 ISH criteria			
	Positive	Negative	Equivocal	Total
Positive	61	0	0	61
Negative	0	409	**6**	415
Equivocal	**9**	**7**	2	18
Total	70	416	8	494

k statistics – 0.838; $p<$**0.001**

In the post-new guideline cases, genomic heterogeneity was detected in 0.47% of the cases (2/422), the proportion of HER2 amplified cells varying between 25 and 40% of the tumor cell population.

3.5 Discussion

Our center (Ipatimup) is one of the reference centers for SISH test of BC in Portugal. In our center, the introduction of the updated ASCO/CAP guideline for HER2 test by SISH resulted in a significant increase of positive cases (12.4% to 16.8%) and decrease of equivocal cases (3.6% to 0.7%).

Several studies recently reported an increase of HER2-positive cases evaluated by FISH but also an increase of HER2-equivocal cases with the introduction of 2013 ASCO/CAP guideline [222-226]. However, the study by the group of Garbar et al. had results similar to ours using FISH, with an increase of HER2-positive cases and a slight decrease in HER2-equivocal cases [227]. The explanation for these differences is not clear, but might be related to the number of cases, pre-analytical conditions, and different ISH platforms. Current guidelines recommend that any specimen used for ER, PgR and HER2 testing should go into fixation immediately (time to fixative within 1 hour), fixed in 10% neutral buffered formalin for 6 to 72 hours and that routine processing is done according to standardized analytically validated protocols [97]. In our study, most cases had incomplete information regarding pre-analytical conditions and we did not review centrally the IHC performed externally, which might explain the decrease in equivocal cases in comparison with recent literature. Nevertheless, the proportion of SISH indeterminate cases was 1.8% (data not shown), which is below published literature [160,228].

As yet, the published concordance rates between SISH and FISH vary between 92 and 99%, the majority fulfilling the ASCO/CAP validation requirement of a concordance rate exceeding 95% (Table 5) [154,158,229-236]. However, the requirement in the 2013 ASCO/CAP guideline to determine the average of HER2 copy number (first applied to bright field ISH and now applied to the FISH test) introduces a problem that did not exist previously.

Autofluorescence in FISH might result in overestimation of both HER2 and CEP17 signals, resulting in HER2/CEP17 ratios below 2.0 and average of HER2 copy numbers above 4 per cell and an increase of equivocal HER2 results [152,237]. If an increase of HER2-equivocal cases by FISH and a decrease of HER2-equivocal cases by SISH is confirmed, the concordance rate of these two ISH tests might decrease to under 95%. This would open up the question which of these techniques provides the most reliable information on HER2 amplification status.

Table 5 Concordance rates of SISH vs FISH according to the 2007 ASCO/CAP guideline

Publication	Concordance (%)	Year
Dietel et al [158]	96	2007
Shousha et al [229]	94	2009
Bartlett et al [230]	96	2009
Papouchado et al [154]	98.9	2010
Koh et al [231]	97	2011
Lee et al [232]	96.7	2011
Park et al [233]	96.5	2012
Jacquemier et al [234]	97	2013
Lim et al [235]	93	2013
Unal et al [236]	92.3	2013

For nearly half of the cases studied (52.1%), both criteria (HER2/CEP17 ratio and average of HER2 copy number per cell) were fulfilled to allow them to be classified as HER2-positive. This is particularly relevant given the fact that half the cases would be excluded from targeted therapy if HER2 amplification would be evaluated using just the HER2 probe (as some methods do).

Classification of the pre-new guideline and post-new guideline case series with the 2007 and 2013 ISH criteria did not result in significant changes in the HER2 test results. This suggests that modifying the threshold in IHC, from 30 to 10% of cells with moderate staining, had little effect on the HER2 amplification test results. Lee et al. found that cases with equivocal IHC (score 2+) in 10-30% of the cells had a probability of being amplified of 5-12% [238]. It is then not surprising that inclusion of these cases does not significantly change the HER2 amplification test results.

In contrast, classification of pre-new guideline and post-new guideline cases with different ISH criteria (2007 and 2013) resulted in significant changes in HER2 amplification test results. Our findings suggest that the 2013 modified ISH criteria had a stronger impact on the test results than the modified IHC criteria. We found that the 2013 ISH criteria reclassified only 4.5% of the cases, leaving more than 95% of the cases with the same classification. Other publications have shown a reclassification rate of up to 15% of cases [223,224].

Polysomy of chromosome 17 changed from 4.1% to 0.9% with the introduction of the 2013 ASCO/CAP guideline, which is probably due to modification of the definition of equivocal IHC HER2 staining (score 2+) rather than a change in the biology of the tumors. Several studies have shown that polysomy of chromosome 17 (measured on the basis of CEP17) varies between 3 and 49% of the cases, depending on the definitions of polysomy and on the method used [155,166,239,240]. The approach is based on the notion that CEP17 copy number is a surrogate marker for chromosome 17 copy number. However, molecular karyotyping has revealed that an increased CEP17 signal number is usually due to gain of the pericentromeric region rather than to duplication of the entire chromosome [241-246]. CEP17 might therefor not be a good marker for polysomy 17, making true polysomy 17 probably a rare event in BC. Nevertheless, CEP17

amplification can still be the cause of misleading HER2 amplification and false-negative test results, excluding patients from anti-HER2 targeted therapy [239].

Tumors with polysomy 17 are thought to be different from non-HER2 amplified tumors, associated with a more aggressive clinical behavior and not responsive to conventional therapy [166,247]. However, in BC, the relationship between polysomy of chromosome 17 and the response to anti-HER2 therapy remains to be determined [248-250].

We found the presence of genomic heterogeneity to be rare as observed in just 0.47% of cases. Several studies have addressed this issue in the past and reported genomic heterogeneity in 5 to 40% of BC cases [165-167,251,252]. Studies on the relationship between genomic heterogeneity and prognosis have shown that tumors with a HER2 amplification in at least 30% of the cells have a reduced disease-free survival [251,252]. However, the definition of genomic heterogeneity has also changed from individual cells (between 5 and 50% of tumor cells with HER2 amplification) to discrete populations of tumor cells (at least 10% of the total tumor cell population with HER2 amplification) [97,162]. Additional work is needed to determine the prevalence of genomic heterogeneity with this new definition and the response to anti-HER2 targeted therapy in these patients.

In conclusion, we show that the new HER2 guideline results in an increased number of HER2-positive and a decreased number of HER2-equivocal cases using the SISH technique, primarily because of modifications of ISH rather than of IHC criteria. As a consequence, the 2013 ASCO/CAP guideline selects more patients for anti-HER2 targeted therapy.

Chapter 4

Counting invasive breast cancer cells in the *HER2* SISH test: how many cells are enough?

(Polonia A et al. *Histopathology.* 2017;71(2):247-257)

4.1 Abstract

Aim: To evaluate the intraobserver and interobserver reproducibility of the HER2 ISH test in breast cancer by measuring the impact of counting different numbers of invasive cancer cells. Methods and results: A cohort of 101 primary invasive breast cancer cases were evaluated for HER2 gene amplification by SISH, and the concordance among four observers with different levels of experience, counting different numbers of invasive cancer cells, was determined. The evaluation of the samples included scoring 20 nuclei, in three different areas. The cases were scored twice, with a washout interval of at least 2 weeks. We observed an increase in the intraobserver concordance rate between the first and second evaluations with an increase in cell count. A count of 60 invasive cells was needed to obtain a concordance rate near 95% and an agreement rate greater than 0.80 by all observers. The interobserver concordance rate of the HER2 test also increased with the increase in cell count, reaching at least a 90% concordance rate with a count of 60 invasive cells. The median variability of both the HER2/CEP17 ratio and the average HER2 copy number between different evaluations decreased with the increase in cell count, being statistically higher in HER2-positive cases. Conclusions: The minimal cell number recommended in current guidelines should be raised to at least 40, and preferably 60, invasive cells. Moreover, cases with amplification levels close to the threshold should be subjected to a dual count from an experienced observer.

4.2 Introduction

HER2 is a transmembrane protein receptor with tyrosine kinase activity, and is amplified and/or overexpressed in approximately 15-20% of invasive breast cancer (BCs) [144-146]. Numerous clinical trials have demonstrated that HER2-targeted therapy improves progression-free survival and overall survival only in patients with BCs showing HER2 amplification [103-106]. For this reason, the accurate assessment of HER2 amplification identifies patients who are most likely to benefit from targeted therapy.

HER2 evaluation is most frequently performed by immunohistochemistry (IHC), resulting in three possible outcomes: negative (score of 0 or 1+), equivocal (score of 2+), and positive (score of 3+). In cases of equivocal results, reflex testing should be performed with *in situ* hybridization (ISH) assays for the assessment of HER2 amplification [97].

Regarding ISH assays, the 2013 American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines accept bright-field ISH and recommend counting at least 20 non-overlapping cells in two separate areas of invasive cancer [97]. Although this is usually interpreted as counting a total of 40 cells (at least 20 cells per area), the supplementary data of the guidelines actually explain that the minimum cell number is, in fact, a total of 20 cells in two separate areas of invasive cancer (at least 10 cells per area). It has already been shown that high ISH interobserver reproducibility exists; nevertheless, the minimum number of cells that should be counted to obtain a reproducible result is yet to be determined [157,158]. Even if the current ASCO/CAP guideline recommendations are adhered to, the imprecision of HER2 testing remains a relevant issue, for both IHC and ISH techniques [159,160].

In the present study, HER2 amplification status was determined in a series of primary BC cases by four different observers, who scored the cases twice according to the 2013 ASCO/CAP guidelines for HER2 testing. Specifically, we aim to evaluate the intraobserver and interobserver interpretative reproducibility of the HER2 assay in BCs by using bright-field ISH to evaluate the impact of counting different numbers of invasive cancer cells.

4.3 Materials and methods

Cases

A cohort of 101 consecutive primary invasive BC cases was retrieved from the archives of Ipatimup Diagnostics from April to June 2015 to determine the concordance of the HER2 amplification assay among four observers counting different numbers of invasive cancer cells.

The cases included formalin-fixed paraffin-embedded core biopsies and surgical specimens referred to our institution with an equivocal HER2 result by IHC (score of 2+) for performance of an evaluation of HER2 amplification with bright-field ISH. All cases were reviewed for diagnosis and histological grade. Ethical approval and informed consent were not required for this study.

Silver ISH

Silver ISH was performed on 3-µm-thick sections with dual-hapten, dual-colour ISH. The dual-probe assay (INFORM HER2 Dual ISH DNA Probe Cocktail Assay; Ventana Medical Systems, Tucson, AZ, USA), which is Food and Drug Administration-approved, contains an HER2 locus-specific probe (black signal) and a control probe specific for the centromere of chromosome 17 [centromere enumeration probe 17 (CEP17), red signal] that allows detection of HER2 amplification by light microscopy. The entire procedure was carried out on an automated staining system (Ventana BenchMark XT Staining System; Ventana Medical Systems, Tucson, AZ, USA) according to the manufacturer's instructions. Appropriated positive and negative controls were used in every set of slides. Optimal staining consists of an absence of non-specific

background staining, distinct nuclear morphology, and clear and specific signals within the nucleus.

SISH interpretation

The samples were classified by two pathologists (A.P. and C.E.) and two pathology residents (J.P. and A.B.) according to the 2013 ASCO/CAP ISH criteria for HER2 amplification. Pathologists had different levels of experience, one with >500 cases evaluated per year (A.P.) and the other with <50 cases evaluated per year (C.E.). Both pathology residents had attended a previous molecular pathology training course, where they learned how to perform SISH interpretation. Corresponding hematoxylin and eosin staining was used for the identification of the invasive component of the tumor, and only cells with a minimum of one copy of HER2 and CEP17 each were scored. The number of HER2 signals was estimated in clusters, except for doublets, which counted as a single signal.

The evaluation of the samples included scoring 20 nuclei, in three different areas, and recording the number of HER2 and CEP17 signals in groups of 10 invasive cancer cells. This approach allows us to add groups of 10 cells in each area to groups of 10 cells in other areas, giving 12 results of scoring 20 cells in two separate areas (10 cells per area) (Figure 1). The same can be applied to the evaluation of scoring 40 cells in two separate areas (20 cells per area, adding the first area to the second and third areas, and the second area to the third area), creating three results. Finally, the three areas can be added, generating one result of scoring 60 cells per case. The cases were scored twice with a washout interval of at least 2 weeks, in a blinded mode.

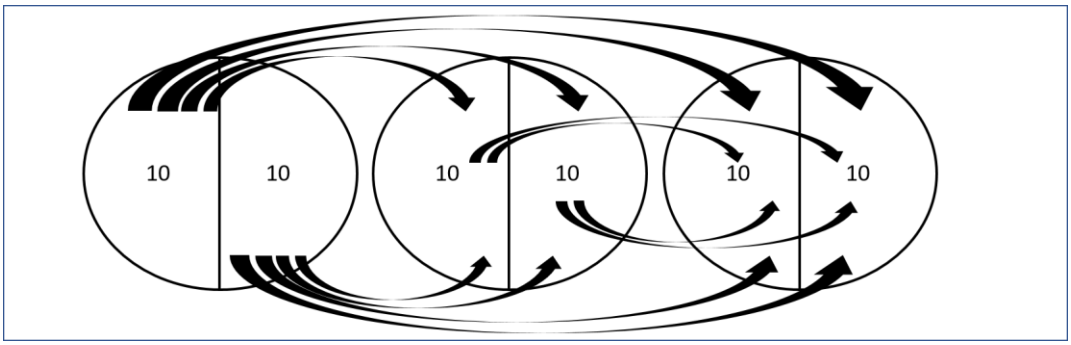


Figure 1 Scoring 20 cells in two separate areas (10 cells per area), giving 12 results

The 2013 ASCO/CAP guidelines establish the result of HER2 amplification as: positive when the HER2/CEP17 ratio is ≥ 2.0 or < 2.0, and the average HER2 copy number is ≥ 6.0 signals per cell; equivocal when the HER2/CEP17 ratio is < 2.0, and the average HER2 copy number is ≥ 4.0 and < 6.0 signals per cell; and negative when the HER2/CEP17 ratio is < 2.0, and the average HER2 copy number is < 4.0 signals per cell [97].

Cases with discordant results were reviewed by both pathologists during a common microscopy session to document genomic heterogeneity, defined in the latest ASCO/CAP guideline as a discrete population of tumor cells with HER2 amplification [97]. No additional testing was performed to resolve the discordance.

Chromosome 17 polysomy was defined as an average of ≥ 3.0 CEP17 signals per cell [166].

Statistical analysis

Statistical analyses were performed with SPSS version 24.0 for Windows. Pearson´s chi-squared (χ^2) test (or Fisher´s exact test, if appropriate) was used for comparison of qualitative variables, and the Mann-Whitney *U*-test (MWU) and Pearson´s correlation coefficient (PCC) were used for comparison of quantitative variables. The level of significance was set at $p < 0.05$.

Agreement rates of the same observer (intraobserver) and those between each observer (interobserver) regarding interpretation of the HER2 amplification assay were evaluated with kappa (*k*) statistics. *k*-values range between zero (chance agreement) and 1 (perfect agreement), and were considered to be satisfactory if they were >0.80 [221].

Altman-Bland analysis was used to assess the agreement between the first and the second measurements of 60 invasive cancer cells. The x-axis represents the mean of the measurements and the y-axis shows the difference between the measurements for each case. Altman-Bland plots display the mean difference (solid line) and 95% agreement limits (dashed lines) in two separate categories: HER2/CEP17 ratio <2.0 and HER2/CEP17 ratio ≥2.0 [253,254]. If there is high agreement between measurements, the differences are expected to be centred around zero, with a narrow agreement limits.

4.4 Results

The cohort included 82 core biopsies and 19 surgical specimens, with 97.03% of the cases being diagnosed in women and 2.97% in men. The age of the patients ranged from 35 to 93 years, with a median age at diagnosis of 65 years. The majority of the histological types were invasive carcinomas of no special type, with 11.88% of the cases being classified as grade 1, 61.39% as grade 2, and 26.73% as grade 3. The cohort characteristics are summarized in Table 1.

Table 1 Cohort characteristics

	Value (n)
Procedure (core biopsy / surgical specimen)	82 / 19
Gender (female / male)	98 / 3
Age (years) (mean ± sd)	63.67±15.77
Histological type	
Invasive carcinoma, NST	94
Lobular carcinoma	5
Mucinous carcinoma	2
Histological grade	
Grade 1 / Grade 2 / Grade 3	12 / 62 / 27

sd – standard deviation; NST – no special type

The final classification of HER2 testing from each observer (first and second evaluations of 60 invasive cancer cells) varied between 23% and 26% of HER2-positive cases (Table 2); there were no cases with genomic heterogeneity. Moreover, no equivocal results or chromosome 17 polysomy were reported by any observer.

Table 2 Classification of HER2 test (first and second evaluation of 60 cells per case)

	Pathologist 1		Pathologist 2		Resident 1		Resident 2	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Positive n (%)	25 (24.8%)	25 (24.8%)	23 (22.8%)	23 (22.8%)	25 (24.8%)	24 (23.8%)	25 (24.8%)	26 (25.7%)
Negative n (%)	76 (75.2%)	76 (75.2%)	78 (77.2%)	78 (77.2%)	76 (75.2%)	77 (76.2%)	76 (75.2%)	75 (74.3%)
Equivocal (n)	0	0	0	0	0	0	0	0
Total (n)	101							

The intraobserver concordance rate of the HER2 test with different cell counts is shown in Table 3. We found, for all observers, an increase in the concordance rate between the first and second evaluations with the increase in cell count from 20 to 60 invasive cells [93.07-100%, 86.14-94.06%, 92.08-99.01% and 76.24-95.05% for pathologist 1 (P1), pathologist 2 (P2), resident 1 (R1) and resident 2 (R2), respectively]. P1 was the only observer who reached an intraobserver agreement rate between the first and second evaluations of >0.80 (0.806) just by counting 20 invasive cells; the same goal was achieved by R1 when counting 40 invasive cells (0.945). Additionally, both P1 and R1 needed to count at least 40 invasive cells to achieve a concordance rate between the first and second evaluations of >95% (97.03% and 98.02%, respectively). On the other hand, P2 and R2 needed to count 60 invasive cells to reach an intraobserver agreement rate of >0.80 (0.831 and 0.869, respectively) and a concordance rate of ~95% (94.06% and 95.05%, respectively). We also found that the concordance rates for different areas within the first or second evaluations were always higher than those measured between different evaluations. The same trend was observed in the intraobserver correlation of both the HER2/CEP17 ratio and the average HER2 copy number, whereby it increased with the increase

in cell count and was always higher for different areas within each evaluation than for different evaluations (Tables S1 and S2).

Table 3 Intraobserver concordance rate of HER2 test results with different cell counts

	Pathologist 1			Pathologist 2			Resident 1			Resident 2		
Cells	1st	2nd	1st vs 2nd	1st	2nd	1st vs 2nd	1st	2nd	1st vs 2nd	1st	2nd	1st vs 2nd
20	96.04 - 100% (0.891 - 1.000)	95.05 - 100% (0.862 - 1.000)	93.07 - 100% (0.806 - 1.000)	93.07 - 100% (0.800 - 1.000)	91.09 - 100% (0.758 - 1.000)	86.14 - 97.03% (0.632 - 0.911)	95.05 - 100% (0.868 - 1.000)	96.04 - 100% (0.891 - 1.000)	92.08 - 99.01% (0.786 - 0.973)	86.14 - 99.01% (0.638 - 0.972)	86.14 - 100% (0.662 - 1.000)	76.24 - 96.04% (0.442 - 0.896)
40	99.01 - 100% (0.973 - 1.000)	98.02 - 100% (0.947 - 1.000)	97.03 - 100% (0.919 - 1.000)	96.04 - 98.02% (0.887 - 0.944)	98.02 - 99.01% (0.945 - 0.972)	90.10 - 95.05% (0.727 - 0.861)	99.01 - 100% (0.973 - 1.000)	100% (1.000)	98.02 - 99.01% (0.945 - 0.973)	94.06 - 97.03% (0.842 - 0.926)	98.02 - 100% (0.949 - 1.000)	90.10 - 95.05% (0.752 - 0.869)
60	na	na	100% (1.000)	na	na	94.06% (0.831)	na	na	99.01% (0.973)	na	na	95.05% (0.869)

Minimal and maximal concordance rate (*k* statistics); na – not applicable

The minimal interobserver concordance rate of the HER2 test increased with the increase in cell count from 20 to 60 invasive cells (73.27% in the P2-R2 evaluation of 20 cells, to 97.03% in the P1-R1 evaluation of 60 cells) (Table 4). A concordance rate of 95.05% and an agreement rate of 0.861 were achieved by P1 and R1 with a count of 40 invasive cells. A count of 60

invasive cells produced a minimal interobserver concordance rate of 90.10% and an agreement rate of 0.734 (R1-R2). In parallel, the same trend described above was found for the correlation of both HER2/CEP17 ratio and average HER2 copy number, in which it increased with the increase in cell count (Tables S3 and S4).

Table 4 Interobserver concordance rate of HER2 test results with different cell counts

	Pathologist 2	Resident 1	Resident 2	Cells
Pathologist 1	87.13-98.02% (0.628-0.945)	92.08-99.01% (0.776-0.974)	77.23-97.03% (0.461-0.921)	20
	91.09-95.05% (0.734-0.865)	95.05-99.01% (0.861-0.973)	88.12-97.03% (0.690-0.921)	40
	93.07-95.05% (0.806-0.865)	97.03-98.02% (0.919-0.947)	92.08-97.03% (0.787-0.921)	60
Pathologist 2		81.19-98.02% (0.515-0.946)	73.27-96.04% (0.386-0.884)	20
		89.11-97.03% (0.704-0.919)	89.11-95.05% (0.695-0.861)	40
		91.09-96.04% (0.750-0.891)	91.09-94.06% (0.751-0.841)	60
Resident 1			77.23-95.05% (0.461-0.869)	20
			88.12-96.04% (0.681-0.894)	40
			90.10-95.05% (0.734-0.869)	60

Minimal and maximal concordance rate (*k* statistics)

The discordant cases of the observers with higher interobserver concordance or agreement rates (P1 and R1) showed an HER2/CEP17 ratio of between 1.39 and 2.72 when 20 invasive cells were counted, of between 1.75 and 2.37 when 40 invasive cells were counted, and of between 1.82 and 2.24 when 60 invasive cells were counted (Table 5; Figure 2). The range of average HER2 copy number in discordant cases for each observer with different cell counts is shown in Table S5. Additionally, we observed at least one discordant case for any observer in 39.02% of core biopsies (32/82) and in 10.53% of surgical specimens (2/19) when 20 invasive cells were counted (χ^2 test; p=0.018), and in 14.63% of core biopsies (12/82) and in 10.53% of surgical specimens (2/19) when 60 invasive cells were counted (Fisher´s exact test; p=1.000).

Table 5 Minimum and maximum HER2/CEP17 ratio in discordant cases with different cell counts

	Pathologist 1			Pathologist 2			Resident 1			Resident 2		
Cells	1st	2nd	1st vs 2nd	1st	2nd	1st vs 2nd	1st	2nd	1st vs 2nd	1st	2nd	1st vs 2nd
20	1.66 - 2.41	1.70 - 2.72	1.66 - 2.72	1.32 - 4.37	1.21 - 4.25	1.21 - 4.37	1.39 - 2.68	1.65 - 2.56	1.39 - 2.68	1.22 - 5.13	0.79 - 3.00	0.79 - 5.13
40	1.98 - 2.05	1.89 - 2.25	1.89 - 2.25	1.57 - 2.13	1.71 - 2.24	0.99 - 3.44	1.94 - 2.15	na	1.75 - 2.37	1.56 - 2.42	1.80 - 2.00	1.56 - 3.32
60	na	na	na	na	na	1.02 - 3.34	na	na	1.82 - 2.24	na	na	1.56 - 2.94

na – not applicable

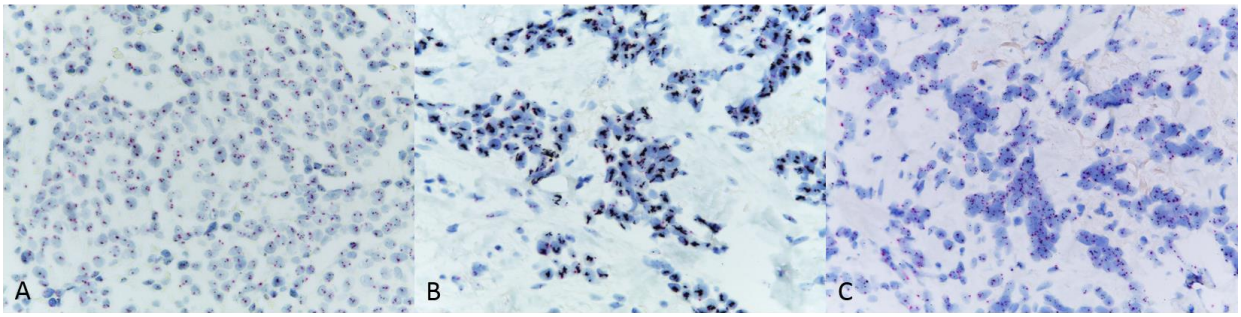


Figure 2 Examples of results of HER2 detection with the SISH technique (×400)

A: HER2 negative for all observers; B: HER2 positive for all observers; C: HER2 discordancy among observers

The median variability of the HER2/CEP17 ratio between different evaluations decreased with the increase in cell count from 20 to 60 invasive cells in both negative cases (0.36-0.06, 0.47-0.15, 0.38-0.08 and 0.54-0.19 for P1, P2, R1 and R2, respectively) and positive cases (1.83-0.50, 1.04-0.31, 1.32-0.27 and 2.44-0.66 for P1, P2, R1 and R2, respectively) (MWU test; $p<0.001$ for all observers). Furthermore, the median variability of the HER2/CEP17 ratio in HER2-positive cases was statistically higher than that in HER2-negative cases (MWU test; $p<0.001$ for all observers) (Table 6).

Table 6 Variability of HER2/CEP17 ratio in negative and positive cases with different cell counts

	Pathologist 1			Pathologist 2			Resident 1			Resident 2			
Cells	1st	2nd	1st- 2nd	1st	2nd	1st-2nd	1st	2nd	1st-2nd	1st	2nd	1st-2nd	Result
20	0.23 (0.06 - 0.77)	0.24 (0.09 - 0.76)	0.36 (0.14 - 1.04)	0.27 (0.11 - 0.73)	0.34 (0.04 - 0.74)	0.47 (0.21 - 1.18)	0.30 (0.11 - 0.56)	0.26 (0.09 - 0.61)	0.38 (0.15 - 0.83)	0.34 (0.05 - 0.86)	0.28 (0.08 - 0.83)	0.54 (0.19 - 1.23)	N
	0.93 (0.39 - 3.86)	1.51 (0.11 - 3.30)	1.83 (0.82 - 5.03)	1.11 (0.37 - 3.50)	0.85 (0.34 - 5.31)	1.04 (0.52 - 5.31)	0.81 (0.17 - 2.38)	0.81 (0.16 - 2.73)	1.32 (0.24 - 4.54)	1.81 (0.46 - 2.60)	1.33 (0.24 - 3.91)	2.44 (1.09 - 4.87)	P
40	0.10 (0.01 - 0.31)	0.08 (0.00 - 0.30)	0.16 (0.04 - 0.78)	0.11 (0.01 - 0.32)	0.13 (0.01 - 0.33)	0.26 (0.08 - 1.10)	0.08 (0.02 - 0.43)	0.07 (0.00 - 0.44)	0.18 (0.03 - 0.67)	0.10 (0.01 - 0.40)	0.07 (0.01 - 0.32)	0.29 (0.05 - 1.01)	N
	0.46 (0.05 - 1.87)	0.30 (0.06 - 1.24)	0.92 (0.21 - 3.17)	0.33 (0.04 - 1.20)	0.33 (0.10 - 0.84)	0.54 (0.18 - 1.07)	0.25 (0.04 - 0.90)	0.22 (0.05 - 1.37)	0.53 (0.08 - 3.48)	0.45 (0.11 - 1.17)	0.38 (0.03 - 1.12)	1.25 (0.27 - 4.03)	P
60	na	na	0.06 (0.00 - 0.63)	na	na	0.15 (0.00 - 0.94)	na	na	0.08 (0.00 - 0.61)	na	na	0.19 (0.00 - 1.08)	N
	na	na	0.50 (0.00 - 2.48)	na	na	0.31 (0.07 - 2.50)	na	na	0.27 (0.01 - 3.03)	na	na	0.66 (0.04 - 3.24)	P

Median (minimum – maximum); N – negative; P – positive; na – not applicable;

20 cells vs 40 cells, and 40 cells vs 60 cells: MWU test $p<0.001$; Negative vs Positive: MWU test $p<0.001$;

1st vs 2nd: MWU test $p>0.05$; 1st vs 1st-2nd, and 2nd vs 1st-2nd: MWU test $p<0.05$

In discordant cases the median variability of the HER2/CEP17 ratio presented values between those of HER2-negative and HER2-positive cases and decreased with the increase in cell count (0.65-0.40, 1.01-0.93, 0.96-0.42 and 1.13-0.63 for P1, P2, R1 and R2, respectively), with the majority being statistically different from negative cases, positive cases, or both (Table 7).

Table 7 Variability of HER2/CEP17 ratio in discordant cases with different cell counts

	Pathologist 1			Pathologist 2			Resident 1			Resident 2		
Cells	1st	2nd	1st- 2nd	1st	2nd	1st-2nd	1st	2nd	1st-2nd	1st	2nd	1st-2nd
20	0.46[2] (0.10 - 0.59)	0.55[1,2] (0.24 - 0.79)	0.65[1,2] (0.52 - 1.11)	0.79[1] (0.31 - 2.65)	0.84[1] (0.39 - 2.58)	1.01[1] (0.39 - 3.45)	0.59[1] (0.36 - 0.77)	0.46[1] (0.26 - 0.66)	0.96[1] (0.43 - 1.36)	0.93[1,2] (0.22 - 3.63)	0.75[1,2] (0.24 - 2.10)	1.13[1,2] (0.52 - 6.76)
40	0.07 (0.07 - 0.07)	0.24[1] (0.17 - 0.30)	0.40[1] (0.30 - 0.69)	0.21[1] (0.15 - 0.53)	0.43[1] (0.33 - 0.53)	0.77[1] (0.18 - 2.01)	0.22 (0.22 - 0.22)	na	0.42 (0.21 - 0.62)	0.34[1] (0.12 - 0.57)	0.17 (0.13 - 0.20)	0.77[1] (0.45 - 1.61)
60	na	na	na	na	na	0.93[1,2] (0.41 - 1.68)	na	na	0.42 (0.42 - 0.42)	na	na	0.63[1] (0.25 - 1.21)

Median (minimum – maximum); na – not applicable; [1]: MWU test $p<0.05$ (comparison with variability of negative cases); [2]: MWU test $p<0.05$ (comparison with variability of positive cases)

In Figure 3 we observe an Altman-Bland analysis (A) for all observers between the first and second evaluations when 60 invasive cells were counted showing narrow agreement limits in cases with HER2/CEP17 ratio <2.0 and wider agreement limits in cases with HER2/CEP17 ratio ≥2.0. Thus, the relationship of the average of the HER2/CEP17 ratio with its variability

confirms the increased variability in cases with a higher HER2/CEP17 ratio (B – PCC 0.881; p<0.001). We also found that, for all observers, the median variability of the HER2/CEP17 ratio between different areas within the first and second evaluations was similar (MWU test; p>0.05) and always lower than the variability between different evaluations (MWU test; p<0.05). The same pattern described above was observed in the variability of the average HER2 copy number with different cell counts in negative, positive and discordant cases (Tables S6 and S7).

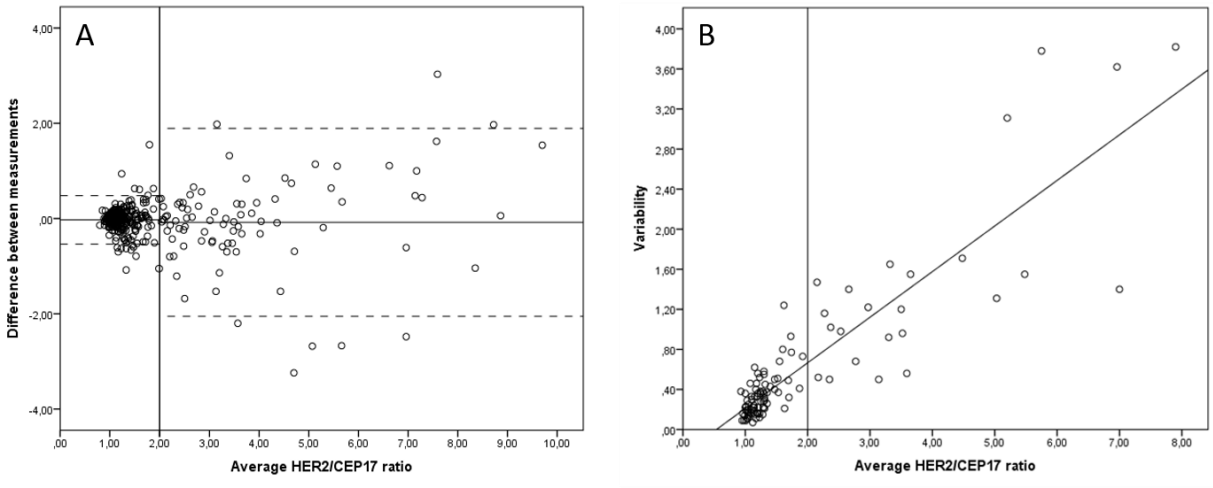


Figure 3 Altman-Bland analysis (A) and relationship of the average of the HER2/CEP17 ratio with its variability (B) between the first and second measurements when 60 invasive cells are counted

Finally, when we compared the variability of the HER2/CEP17 ratio with the variability of the average HER2 copy number, we observed that the former was always inferior to the latter (MWU test; p<0.05 for all observers when counting 60 invasive cells) (Tables 6 and S6).

4.5 Discussion

Since the introduction of the new ASCO/CAP guidelines for HER2 testing in BC, numerous studies have reported an increase in the number of HER2-positive cases [222-226]. Recently, using SISH, we showed that the updated ASCO/CAP guidelines resulted in a significant increase in the number of HER2-positive cases and a decrease in the number of equivocal cases [255]. Most of the published literature shows the concordance rates between SISH and fluorescent ISH to be >90%, and to almost always fulfil the ASCO/CAP validation requirement of a concordance rate of >95% [154,231,234].

In the present study, we found an increase in the concordance rate of HER2 testing between the first and second evaluations with an increase in cell count from 20 to 60 invasive cells, as well as a decrease in the variability of HER2/CEP 17 ratios for all observers, which demonstrated the value of counting additional cells. The fact that only the observer with more experience (P1) could achieve an intraobserver agreement rate of >0.80 between both evaluations when counting only 20 invasive cells shows that 20 cells might not be the optimal minimal cell number as recommended by current guidelines. In fact, P1 and R1 needed to count at least 40 invasive cells to reach an intraobserver concordance rate of >95%, and P2 and R2 needed to count 60 invasive cells to achieve the same rate. Additionally, no observer reached an interobserver concordance rate of >95% or an agreement rate of at least 0.80 by counting only 20 invasive cells. In fact, only two observers (P1 and R1) achieved that aim, and required a minimal count of 40 invasive cells. The remaining observers required a count of 60 invasive cells to achieve concordance rates of >90% of the cases and agreement rates near 0.80. According to our data, the minimal cell number of 20 invasive cells in HER2 testing in BC is not sufficient, and should be raised to at least 40 invasive cells (which is probably already the case in most

laboratories) and, preferably, 60 cells if available, which is almost always manageable in most of the cases in our experience.

Interestingly, the observers with higher interobserver concordance/agreement rates were those with better intraobserver concordance/agreement rates. Additionally, the major causes of discordance in these observers were tumors with an HER2/CEP17 ratio near the threshold, even when 60 invasive cells were counted. Given that heterogeneity was ruled out, the discordant cases of the observers with lower performance were probably attributable to counting in non-cancer areas or in areas with excessive background, showing a wider range of HER2/CEP17 ratios. ISH analysis should start with scanning of the entire slide prior to counting, and compare with IHC to define the areas of potential HER2 amplification. Furthermore, the absence of information on preanalytical conditions can jeopardize the execution of the ISH technique and create artefacts that impair proper measurement of the signals. Although chromosome 17 polysomy can cause impairment of ISH interpretation, it was not an issue in this study, given that no observer found such cases.

Unexpectedly, we found more discordant cases in core biopsies than in surgical specimens. Although counting additional cells eliminates this problem, we think that it is probably related to the higher quantity of tumor in surgical specimens, which allows for the identification of better scoring areas, with less background and fewer overlapping cells. Previously, it has been shown that the identification of amplification status by SISH is very robust, as consensus between several observers cannot be reached in <2% of cases, although a consensus in discordant cases was not the aim in this study [154].

As previously shown, the cases with amplification levels close to the thresholds are the most likely to be discordant [256]. For cases in which the HER2/CEP17 ratio lies between 1.80 and

2.20, the ASCO/CAP guidelines recommend that a different person counts an additional 20 invasive cells, whereas the updated UK guidelines recommend counting at least 60 invasive cells (preferably with a dual count by a second observer) [97,257]. We agree with the UK guidelines recommendation, because 20 additional invasive cells evaluated by a different observer will not be sufficient to assess doubtful cases with an HER2/CEP17 ratio around 2.0, as we show in this study. Moreover, the similar variability of the HER2/CEP17 ratio within each evaluation, along with the divergent measurements between the first and second evaluations, suggests that the observers have the same error but different bias in different evaluations. We therefore recommend counting additional cells immediately than counting on a different day.

The UK guidelines for HER2 assessment in BC recommend that, in cases with either clear amplification or an HER2/CEP17 ratio lower than 1.5, scoring of 20 tumor cells is sufficient [257]. Although our data support this recommendation for experienced observers, it is more practical to immediately count 40 tumor cells and calculate both the HER2/CEP17 ratio and the average HER2 copy number than to count 20 cells, calculate these values, and then decide to count additional cells.

Training and experience in the interpretation of HER2 ISH testing is essential. The UK guidelines recommend that laboratories perform at least 100 HER2 ISH assays every year, and, when new personnel are being trained in the interpretation of HER2 testing, observations of at least 100 ISH tests in parallel with an experienced scorer should be performed until a minimum concordance of 95% is achieved [257]. In our study, although both residents had the same level of experience after training, only one reached a concordance rate of at least 95% with the experienced observer, which underlines the importance of validation studies in this field.

The variability of both the HER2/CEP17 ratio and of the average HER2 copy number in HER2-positive cases were always statistically higher than in HER2-negative cases, probably because of the different estimation of the number of HER2 signals in clusters. Another explanation could be the genetic instability of HER2 amplification already described between primary BC and metastatic lesions in HER2-positive cases, whereby the later had a significant increase in HER2 copy number [258]. In the same way, separate areas of HER2-positive tumors could, in fact, have different HER2/CEP17 ratios and HER2 copy numbers, although this needs to be clarified in future studies. Recently, it has even been shown that the variability of the HER2/CEP17 ratio and of the average HER2 copy number between manual and automatic procedures using imaging analysis is higher in HER2-positive BC cases, although the results in separate areas were not compared in each procedure [259]. Finally, the lower variability of the HER2/CEP17 ratio than of the HER2 copy number shows that the ratio value is probably more reproducible among observers, probably because the larger size of the CEP17 signal makes it easy to identify [154,260]. Although the ISH technique is the gold standard for identifying HER2-positive BC cases because it evaluates HER2 amplification only in invasive cancer cells, it might not be the most accurate method for HER2 quantification. One of the first studies investigating the effects of the level of HER2 amplification showed that high-amplification tumors had a significantly higher rate of pathologically complete response to neoadjuvant treatment with trastuzumab than low-amplification tumors [261]. However, the same authors showed later that HER2 amplification level was not correlated with either recurrence-free or overall survival in the same setting [262]. Moreover, the data from the Herceptin Adjuvant (HERA) trial were also used to compare the degree of HER2 amplification with clinical outcome in HER2-positive BC (in both the untreated and trastuzumab-treated arms) [263]. Both the HER2/CEP17 ratio and the HER2 copy

number were measured, and no significant effect on prognosis or benefit from trastuzumab was observed with different levels of HER2 amplification. In contrast, it was recently reported that there was an increased risk of death from BC in the first 5 years after diagnosis in women with HER2 copy numbers of ≥ 6, as well as of ≥ 4.0 and < 6.0, irrespective of HER2/CEP17 ratio [264]. The conflicting clinical data suggest that the variability of the HER2/CEP17 ratio and the HER2 copy number, depending on the number of cells counted, may determine the accuracy of the final result. Accordingly, to evaluate the putative clinical implications of the degree of HER2 amplification, alternative methodology that quantifies HER2 amplification more precisely must be tested. Therefore, imaging analysis of ISH tests, counting several hundreds of cells more objectively, and HER2 quantification by molecular techniques in separate areas of invasive carcinoma should be investigated to assess the variability of the results in HER2-positive BC cases.

In conclusion, we show that counting 20 cells in the HER2 ISH test is not sufficient to obtain a reproducible result, and that the minimal cell number should be raised to at least 40, and preferably 60, invasive BC cells. Additionally, cases with amplification levels close to the threshold should have a count of at least 60 cells, and, if possible, a dual count from an experienced observer. As far as we know, this is the first time that SISH results have been compared between multiple observers counting different numbers of invasive cancer cells.

4.6 Supplementary information

Table S1 Minimal and maximal intraobserver correlation of the HER2/CEP17 ratio with different cell counts

	Pathologist 1			Pathologist 2			Resident 1			Resident 2		
Cells	1st	2nd	1st vs 2nd	1st	2nd	1st vs 2nd	1st	2nd	1st vs 2nd	1st	2nd	1st vs 2nd
20	0.951	0.964	0.909	0.897	0.717	0.690	0.972	0.963	0.947	0.889	0.915	0.746
	-	-	-	-	-	-	-	-	-	-	-	-
	0.999	0.995	0.982	0.988	0.988	0.936	0.996	0.995	0.976	0.990	0.988	0.929
40	0.987	0.991	0.945	0.985	0.982	0.880	0.994	0.992	0.968	0.982	0.989	0.867
	-	-	-	-	-	-	-	-	-	-	-	-
	0.991	0.993	0.983	0.987	0.993	0.913	0.997	0.997	0.976	0.990	0.992	0.890
60	na	na	0.966	na	na	0.904	na	na	0.977	na	na	0.882

Pearson´s correlation coefficient: $p<0.001$ for all correlations; na – not applicable

Table S2 Minimal and maximal intraobserver correlation of the average HER2 copy number with different cell counts

	Pathologist 1			Pathologist 2			Resident 1			Resident 2		
Cells	1st	2nd	1st vs 2nd	1st	2nd	1st vs 2nd	1st	2nd	1st vs 2nd	1st	2nd	1st vs 2nd
20	0.964	0.963	0.894	0.926	0.694	0.623	0.985	0.985	0.937	0.891	0.889	0.733
	-	-	-	-	-	-	-	-	-	-	-	-
	0.999	0.991	0.962	0.987	0.987	0.908	0.998	0.997	0.969	0.976	0.987	0.893
40	0.987	0.987	0.922	0.988	0.986	0.811	0.996	0.996	0.950	0.980	0.992	0.846
	-	-	-	-	-	-	-	-	-	-	-	-
	0.994	0.989	0.958	0.990	0.991	0.858	0.998	0.997	0.971	0.988	0.994	0.869
60	na	na	0.946	na	na	0.838	na	na	0.963	na	na	0.864

Pearson´s correlation coefficient: $p<0.001$ for all correlations; na – not applicable

Table S3 Minimal and maximal interobserver correlation of the HER2/CEP17 ratio with different cell counts

	Pathologist 2	Resident 1	Resident 2	Cells
Pathologist 1	0.663-0.940	0.876-0.971	0.739-0.954	20
	0.787-0.933	0.910-0.966	0.823-0.951	40
	0.809-0.917	0.921-0.963	0.853-0.943	60
Pathologist 2		0.614-0.933	0.568-0.951	20
		0.748-0.930	0.765-0.946	40
		0.781-0.923	0.814-0.945	60
Resident 1			0.716-0.955	20
			0.781-0.948	40
			0.796-0.938	60

Pearson´s correlation coefficient: $p<0.001$ for all correlations

Table S4 Minimal and maximal interobserver correlation of the average HER2 copy number with different cell counts

	Pathologist 2	Resident 1	Resident 2	Cells
Pathologist 1	0.657-0.933	0.835-0.964	0.794-0.903	20
	0.763-0.932	0.861-0.952	0.842-0.900	40
	0.805-0.931	0.876-0.946	0.874-0.900	60
Pathologist 2		0.593-0.925	0.454-0.919	20
		0.706-0.921	0.718-0.924	40
		0.729-0.919	0.737-0.921	60
Resident 1			0.738-0.932	20
			0.779-0.926	40
			0.802-0.925	60

Pearson´s correlation coefficient: $p<0.001$ for all correlations

Table S5 Minimum and maximum average HER2 copy number in discordant cases with different cell counts

	Pathologist 1			Pathologist 2			Resident 1			Resident 2		
Cells	1st	2nd	1st vs 2nd	1st	2nd	1st vs 2nd	1st	2nd	1st vs 2nd	1st	2nd	1st vs 2nd
20	2.45 - 3.50	2.00 - 4.35	2.00 - 4.35	2.45 - 8.40	1.70 - 6.20	1.70 - 8.40	2.70 - 4.40	2.45 - 4.20	2.45 - 4.40	1.85 - 6.10	1.70 - 5.40	1.70 - 7.15
40	2.73 - 3.18	2.23 - 3.20	2.23 - 3.20	2.70 - 4.10	2.33 - 3.25	2.08 - 6.38	2.98 - 3.18	na	2.50 - 3.18	2.03 - 4.80	2.00 - 4.00	2.00 - 6.05
60	na	na	na	na	na	2.08 - 5.78	na	na	2.67 - 2.80	na	na	2.10 - 5.38

na – not applicable

Table S6 Variability of the average HER2 copy number in negative and positive cases with different cell counts

	Pathologist 1			Pathologist 2			Resident 1			Resident 2			
Cells	1st	2nd	1st- 2nd	1st	2nd	1st-2nd	1st	2nd	1st-2nd	1st	2nd	1st-2nd	Result
20	0.30 (0.05 - 1.00)	0.30 (0.10 - 1.25)	0.45 (0.20 - 1.70)	0.40 (0.05 - 0.90)	0.50 (0.15 - 1.10)	0.70 (0.35 - 1.50)	0.28 (0.05 - 0.75)	0.25 (0.10 - 0.60)	0.40 (0.15 - 0.90)	0.50 (0.10 - 1.55)	0.35 (0.00 - 1.35)	0.80 (0.20 - 2.00)	N
	1.28 (0.45 - 3.50)	1.40 (0.30 - 4.10)	2.10 (1.20 - 7.20)	1.15 (0.40 - 4.30)	1.13 (0.50 - 5.40)	1.50 (0.80 - 5.40)	1.00 (0.25 - 3.65)	1.08 (0.25 - 2.45)	1.50 (0.40 - 7.25)	1.93 (0.30 - 4.50)	1.18 (0.10 - 4.60)	2.95 (0.70 - 7.15)	P
40	0.14 (0.02 - 0.53)	0.10 (0.00 - 0.38)	0.25 (0.10 - 1.15)	0.15 (0.00 - 0.63)	0.15 (0.05 - 0.38)	0.40 (0.17 - 1.15)	0.10 (0.00 - 0.48)	0.08 (0.00 - 0.45)	0.23 (0.03 - 2.22)	0.15 (0.02 - 1.15)	0.10 (0.00 - 0.55)	0.47 (0.13 - 1.55)	N
	0.61 (0.15 - 2.10)	0.53 (0.10 - 2.20)	1.10 (0.35 - 5.70)	0.48 (0.13 - 1.30)	0.33 (0.05 - 1.08)	0.73 (0.32 - 1.35)	0.27 (0.05 - 1.40)	0.25 (0.03 - 1.38)	0.55 (0.22 - 5.88)	0.50 (0.03 - 1.75)	0.32 (0.02 - 1.23)	1.50 (0.40 - 4.42)	P
60	na	na	0.12 (0.00 - 0.99)	na	na	0.25 (0.03 - 0.95)	na	na	0.12 (0.00 - 1.97)	na	na	0.36 (0.05 - 2.12)	N
	na	na	0.58 (0.05 - 4.34)	na	na	0.42 (0.05 - 3.14)	na	na	0.39 (0.02 - 5.35)	na	na	0.95 (0.15 - 3.97)	P

Median (minimum – maximum); N – negative; P – positive; na – not applicable;

20 cells vs 40 cells, and 40 cells vs 60 cells: MWU test $p<0.001$; Negative vs Positive: MWU test $p<0.001$;

1st vs 2nd: MWU test $p>0.05$; 1st vs 1st-2nd, and 2nd vs 1st-2nd: MWU test $p<0.05$

Table S7 Variability of the average HER2 copy number in discordant cases with different cell counts

	Pathologist 1			Pathologist 2			Resident 1			Resident 2		
Cells	1st	2nd	1st- 2nd	1st	2nd	1st-2nd	1st	2nd	1st-2nd	1st	2nd	1st-2nd
20	0.43[2] (0.20 - 0.80)	0.73[1] (0.25 - 1.55)	0.90[1,2] (0.65 - 1.55)	1.20[1] (0.35 - 4.45)	0.85 (0.30 - 3.20)	1.58[1] (0.55 - 6.15)	0.75[1] (0.25 - 1.00)	0.45[1,2] (0.40 - 0.70)	1.08[1] (0.60 - 2.55)	1.15[1,2] (0.30 - 2.70)	0.70[1] (0.15 - 2.80)	1.38[1,2] (0.50 - 5.90)
40	0.45 (0.45 - 0.45)	0.39[1] (0.38 - 0.40)	0.50[1,2] (0.48 - 0.52)	0.22 (0.10 - 0.50)	0.23 (0.10 - 0.35)	1.14[1] (0.35 - 3.60)	0.20 (0.20 - 0.20)	na	0.31 (0.22 - 0.40)	0.59[1] (0.05 - 1.35)	0.24 (0.20 - 0.28)	0.88[1] (0.25 - 3.15)
60	na	na	na	na	na	1.53[1,2] (0.84 - 2.93)	na	na	0.13 (0.13 - 0.13)	na	na	0.63[1] (0.30 - 2.35)

Median (minimum – maximum); na – not applicable; [1]: MWU test $p<0.05$ (comparison with variability of negative cases); [2]: MWU test $p<0.05$ (comparison with variability of positive cases)

Chapter 5

Characterization of HER2 gene amplification heterogeneity in invasive and *in situ* breast cancer using bright-field ISH

(Polonia A et al. *Virchows Arch.* 2017;471(5):589-598)

5.1 Abstract

The aims of this study were to evaluate and compare the HER2 gene amplification *status* in invasive and adjacent in situ breast carcinoma, using bright-field in situ hybridization, and to document the possible presence of HER2 genetic heterogeneity (HER2-GH) in both components. A cohort of 100 primary invasive carcinomas (IC) associated with carcinoma in situ (CIS) were evaluated for HER2 gene amplification by SISH according to the 2013 ASCO/CAP HER2 guideline. A second cohort of all the cases with HER2-GH since the introduction of the updated ASCO/CAP HER2 guideline was also characterized, and an evaluation of the HER2 gene amplification in the CIS component, if present, was also done. In the first cohort, the HER2 amplification in the IC was negative in 87% of the cases and positive in 13% of the cases, without the presence of HER2-GH. All the cases had an associated CIS with the same HER2 *status* as IC, with four cases of CIS presenting HER2-GH. In the CIS, we observed a significant relationship of HER2 gene amplification with high nuclear grade. In the four cases with HER2-GH in CIS, two cases presented HER2 gene amplification in the IC. The second cohort included 12 cases with HER2-GH in a total of 1243 IC cases (0.97%). Additionally, we identified two cases associated with non-amplified CIS. HER2-GH is a rare event in IC and can already be present in CIS, not being an important step in the acquisition of invasive features.

5.2 Introduction

Human epidermal growth factor receptor 2 (HER2) is amplified and/or overexpressed in about 15 to 20% of invasive breast cancer (BC), being associated with worse clinical outcome and predictive of benefit from HER2-targeted therapy [104,141,148]. The incidence rate of carcinoma in situ (CIS) of the breast, the immediate precursor of invasive carcinoma (IC), has stabilized since the beginning of the millennium in women older than 50 years, but continues to increase about 2% every year in younger women [219]. Several studies have shown that HER2 amplification can already be present in CIS and that frequently HER2 *status* is concordant with the invasive component [142,143].

Heterogeneity has been notice in almost all types of cancer, including BC, being related to several aspects of disease progression and clinical outcome [161]. The first recommendation regarding HER2 genetic heterogeneity (HER2-GH) was published in 2009 as an extension of the 2007 American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) HER2 guidelines after the acknowledgment that some tumors displayed intratumoral heterogeneity and such cases could originate discrepant results between immunohistochemistry (IHC) and in situ hybridization (ISH) analysis [162]. At that time, HER2-GH was defined as HER2 gene amplification in 5 to 50% of invasive cancer cells. Importantly, the definition was based on studies that did not include clinical outcome, being the first step to investigate the clinical significance of HER2-GH and the possible role of target therapy in this setting [163,164]. Thereafter, numerous studies have shown that HER2-GH could be present in BC from 5 to 40% of the cases [165,166]. Additionally, it was shown that HER2-GH was more frequent in cases near the threshold

of positivity and that heterogeneity measured in individual cells is not informative of clonal heterogeneity within a tumor population [167].

Currently, the definition of HER2-GH has changed from individual cells to discrete population of tumor cells with HER2 gene amplification. According to the 2013 ASCO/CAP HER2 guideline, a tumor is considered HER2 positive if HER2 gene amplification is present in at least 10% of the total tumor cell population [97].

The aims of the present study are to compare the HER2 gene amplification *status* in invasive and adjacent in situ BC, using bright-field ISH, and to document the possible presence of heterogeneity in both components.

5.3 Materials and methods

Case selection

The cases included formalin-fixed, paraffin-embedded needle core biopsies (NCB) and surgical excision specimens (SES) referred to Ipatimup Diagnostics with an equivocal HER2 result (score of 2+) in IC by IHC for performance of an evaluation of HER2 amplification with bright-field ISH. There was no information regarding patient treatment.

The first cohort included 100 primary invasive BC cases associated with CIS retrieved from the archives from November 2015 to July 2016 to determine the concordance of HER2 gene amplification in both components. During this period, 347 cases with an equivocal HER2 result by IHC were evaluated for HER2 gene amplification. The cohort comprised 66 NCB and 34 SES, all diagnosed in women. The age of the patients ranged from 31 to 83 years old, with a median age at diagnosis of 54 years. The majority of the histological types were invasive carcinomas of no special type (NST), with 14% of the cases being classified as grade 1, 66% as grade 2, and 20% as grade 3 (Table 1).

Table 1 Characteristics of the first cohort

Procedure (NCB / SES)	66 / 34
Gender (female / male)	100 / 0
Age (mean ± SD)	54.68 ± 12.41
Invasive carcinoma	
Histological type	
Invasive carcinoma, NST	88
Lobular carcinoma	8
Micropapillary carcinoma	2
Mucinous carcinoma	1
Encapsulated papillary carcinoma	1
Histological grade	
Grade 1 / Grade 2 / Grade 3	14 / 66 / 20
HER2 gene amplification	
ISH negative/ISH positive	87/13
Carcinoma in situ	
Ductal	93
Lobular	8
Nuclear grade	
Low / Intermediate / High	3 / 42 / 56
Necrosis (absent / present)	58 / 43
Microcalcifications (absent / present)	73 / 28

NCB: needle core biopsy; SES: surgical excision specimen; SD: standard deviation; NST: no special type

The second cohort included all cases with HER2-GH (primary invasive or metastatic BC) since the introduction of the 2013 ASCO/CAP HER2 guideline (November 2013) to October 2016. An evaluation of HER2 gene amplification in the CIS component, if present, was also done. The cohort comprised 10 NCB and 2 SES with HER2-GH in a total of 1243 cases (0.97%), 11 of which were primary invasive BC and one lymph node metastasis. The age of the patients ranged from 42 to 74 years old, with a median age at diagnosis of 58 years, and two cases were

diagnosed in men. All histological types but one were IC, NST, with eight cases being classified as grade 2 and four cases as grade 3 (Table 2).

Table 2 Characteristics of the second cohort

Procedure (NCB / SES)	10 / 2
Gender (female / male)	10 / 2
Age (mean ± SD)	59.67 ± 10.92
Invasive carcinoma	
Histological type	
Invasive carcinoma, NST	11
Lobular carcinoma	1
Histologic grade	
Grade 1 / Grade 2 / Grade 3	0 / 8 / 4
Carcinoma in situ	
Ductal	2
Nuclear grade	
Low / Intermediate / High	0 / 1 / 1
Necrosis (absent / present)	1 / 1
Microcalcifications (absent / present)	2 / 0

NCB: needle core biopsy; SES: surgical excision specimen; SD: standard deviation; NST: no special type

All cases were reviewed for histological type and grade (Nottingham Histologic Score) in the IC. The characterization of the CIS included nuclear grade, the presence of necrosis, and microcalcifications.

This study has been performed in accordance with the national regulative law for the handling of biological specimens from tumor banks, being the samples exclusively available for research purposes in retrospective studies, as well as under the international Helsinki declaration.

Silver in situ hybridization

SISH technique was performed on 3-µm-thick sections in one block of each case with dual-hapten, dual-colour ISH. The dual-probe assay (INFORM HER2 Dual ISH DNA Probe Cocktail Assay; Ventana Medical Systems, Inc., Tucson, AZ, USA), which is Food and Drug Administration-approved, contains an HER2 locus-specific probe (black signal) and a control probe specific for the centromere of chromosome 17 (centromere enumeration probe-CEP17, red signal), which allows detection of HER2 gene amplification by light microscopy. The entire procedure was carried out on an automated staining system (Ventana BenchMark XT Staining System; Ventana Medical Systems, Inc., Tucson, AZ, USA) according to the manufacturer's instructions. Appropriated positive and negative controls were used in every set of slides.

SISH interpretation

The evaluation of the samples included scoring of at least 20 nuclei, in two different areas, recording the number of HER2 and CEP17 signals. Corresponding hematoxylin and eosin (H&E) staining was used for the identification of the invasive and in situ components of the tumor, and only cells with a minimum of one copy of HER2 and CEP17 each were scored. The number of HER2 signals was estimated in clusters, except for doublets which counted as a single signal. The samples were classified by a pathologist (AP) according to the 2013 ASCO/CAP ISH criteria for HER2 gene amplification: positive when the HER2/CEP17 ratio is ≥ 2.0 or < 2.0 and the average HER2 copy number is ≥ 6.0 signals *per* cell; equivocal when the HER2/CEP17 ratio is < 2.0 and the average HER2 copy number is ≥ 4.0 and < 6.0 signals *per* cell; and negative when the HER2/CEP17 ratio is < 2.0 and the average HER2 copy number is < 4.0 signals *per* cell [97].

HER2-GH is defined as tumors with discrete population of tumor cells with different HER2 gene *status* [97]. The proportion of amplified areas was quantified by measuring the number of fields (power field of 200x) with HER2 gene amplification divided by the number of fields of invasive or in situ carcinoma.

Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 24.0 for Windows. The Pearson´s chi-squared (χ^2) test (or the Fisher´s exact test, if appropriate) was used for comparison of qualitative variables and the Mann-Whitney U (MWU) test, the *t* test, and Pearson´s correlation coefficient (PCC) were used for comparison of quantitative variables. The level of significance was set at $p < 0.05$.

5.4 Results

In the first cohort, the HER2 amplification in the IC was negative in 87% of the cases and positive in 13% of the cases, without the presence of HER2-GH. All the cases had an associated CIS with the same HER2 *status* than IC, with four cases of CIS presenting HER2-GH. Because one case presented both lobular and ductal CIS, we characterized 101 types of CIS, where 92.1% were ductal CIS (DCIS) and 2.9% of the cases were classified as low grade, 41.6% as intermediate grade, and 55.5% as high grade. We also observed necrosis in 42.6% and microcalcifications in 27.7% (Table 1).

HER2 amplification in the IC were not related with the procedure, the age of the patients or the histological grade. However, in the CIS, we observed a significant relationship of HER2 amplification with high nuclear grade (18.9% vs 4.4%; p=0.030), without an association with the remaining characteristics (Table 3).

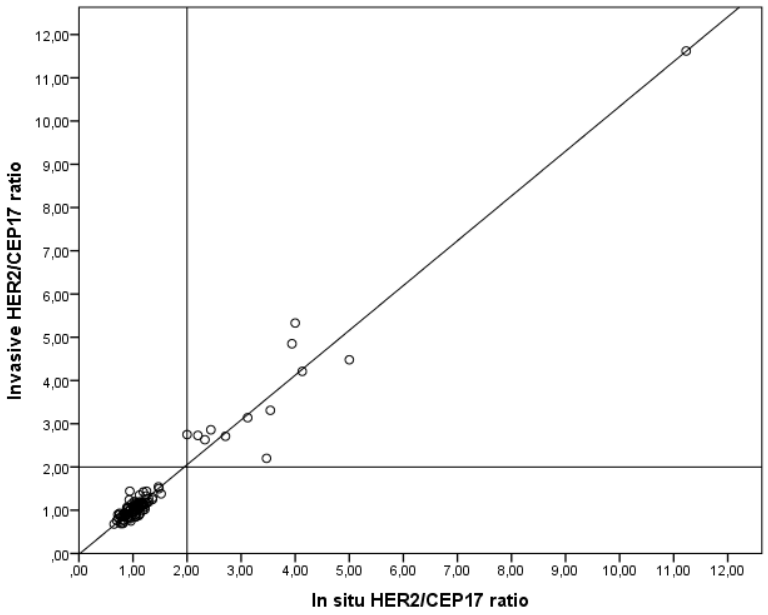
Table 3 HER2 amplification in invasive and in situ carcinoma

Invasive carcinoma	Negative	Positive	*p*
Procedure (NCB/SES)	58/29	8/5	0.759[1]
Age (mean ± SD)	54.52±12.86	55.69±9.29	0.753[2]
Histological grade (Grade 1-2/Grade 3)	72/15	8/5	0.129[1]
Carcinoma *in situ*			
Ductal / Lobular	78/8	12/0	0.590[1]
Nuclear grade (low-intermediate/high)	43/43	2/10	**0.030**[3]
Necrosis (absent/present)	52/34	5/7	0.216[3]
Microcalcifications (absent/present)	65/21	6/6	0.085[1]

NCB: needle core biopsy; SES: surgical excision specimen; SD: standard deviation; [1] Fisher´s exact test; [2] *t*-test; [3] Pearson´s Chi-Squared test

The distribution of HER2/CEP17 ratio and average of HER2 and CEP17 copy number *per* cell were not statistically different between the IC and CIS (Table S1). Additionally, we observed a high correlation of HER2/CEP17 ratio and average HER2 copy number *per* cell between IC and CIS (PCC=0.981; $p<0.001$ and PCC=0.929; $p<0.001$, respectively) (Figure 1 and S1).

Figure 1 Relationship between HER2/CEP17 ratio of in situ and invasive carcinoma



In the four cases with HER2-GH in CIS, only one case was identified in NCB and two cases presented HER2 gene amplification in the IC (Figure 2 and 3). The proportion of cells with HER2 gene amplification in the CIS varied between 30 and 60% of the total CIS represented in the sample, being all high-grade DCIS (for details see Table 4 and S2).

Figure 2
Carcinoma in situ with HER2 genetic heterogeneity associated with HER2-positive invasive carcinoma

Case 1 (1st cohort): A1 - DCIS, H&E 200x; A2 - Amplified DCIS (left) and non-amplified DCIS (right), SISH 200x; B1 - Invasive carcinoma, NST, H&E 200x; B2 - Amplified invasive carcinoma, NST, SISH 200x.

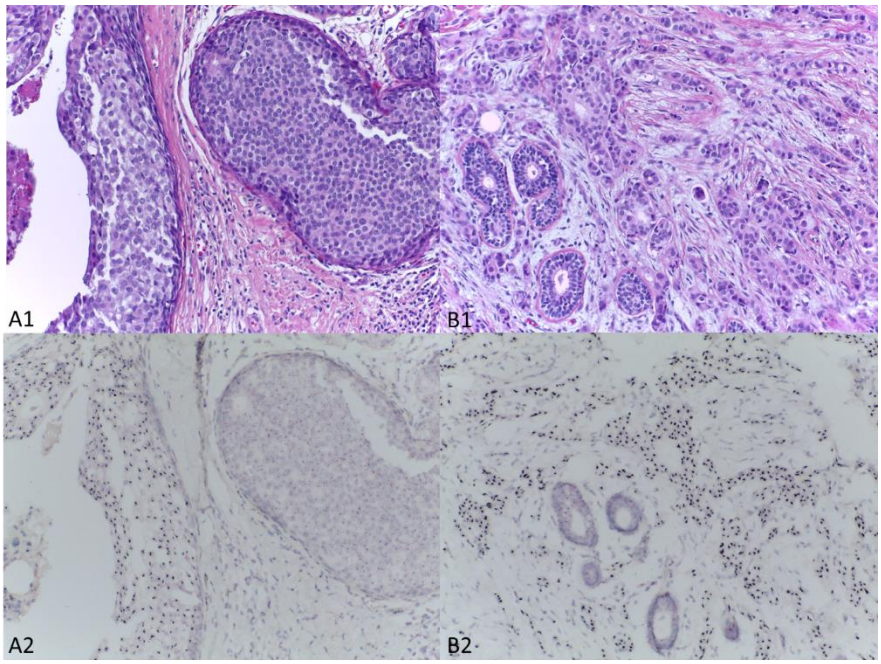


Figure 3
Carcinoma in situ with HER2 genetic heterogeneity associated with HER2-negative invasive carcinoma

Case 4 (1st cohort): A1 - LCIS, H&E 200x; A2 – Non-amplified LCIS, SISH 400x; B1 - Invasive lobular carcinoma, H&E 200x; B2 – Non-amplified invasive lobular carcinoma, SISH 400x; C1 – DCIS, H&E 200x; C2 – Amplified DCIS, SISH, 400x.

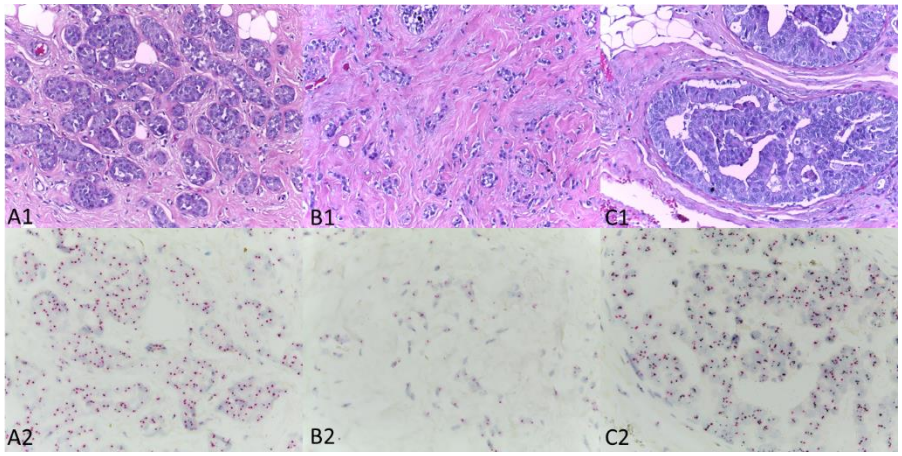


Table 4 Cases with HER2 amplification discordance between invasive carcinoma and carcinoma in situ

Case	Gender	Age	Procedure	IC	Histologic grade	HER2 status	CIS	Nuclear grade	HER2 status	Proportion
1	F	44	SES	NST	2	A	ductal	high	A	40%
									NA	60%
2	F	53	SES	NST	3	A	ductal	high	A	60%
									NA	40%
3	F	48	NCB	NST	3	NA	ductal	high	NA	40%
									A	60%
4	F	78	SES	Lobular	1	NA	lobular	low	NA	70%
							ductal	high	A	30%

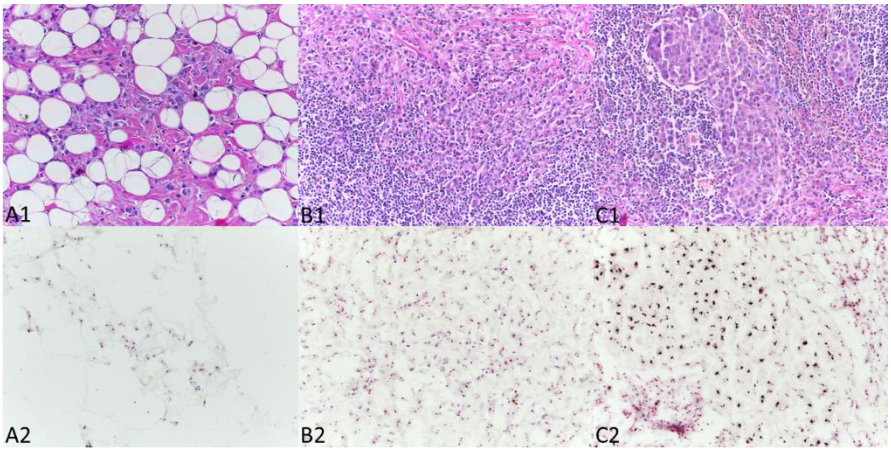
F: female; SES: surgical excision specimen; NCB: needle core biopsy; NST: no special type; A: amplified; NA: not amplified

In the second cohort, the proportion of cells with HER2 amplification varied between 1 and 50% of the total tumor cell population represented in the sample. In the negative component, HER2/CEP17 ratio varied between 1.00 and 1.52, and in the positive component between 2.06 and 9.17 (Table S3).

The primary IC cases had similar morphological features in the amplified and non-amplified components. The lymph node metastasis case represented a primary lobular carcinoma of the breast with an equivocal HER2 result (score of 2+) by IHC and no HER2 gene amplification by SISH (case 2). The metastasis showed two types of neoplastic cells, one similar with the primary lesion and another one with more pleomorphic nuclei. After additional IHC in the metastasis, it was found that the latter areas showed strong and complete membranous staining in 40% of the cells (score of 3+) that were confirmed by SISH as HER2 amplified (Figure 4).

Figure 4
HER2-negative invasive carcinoma associated with HER2 genetic heterogeneity in the lymph node metastasis

Case 2 (2nd cohort): A1 – Invasive lobular carcinoma, H&E 200x; A2 – Non-amplified invasive lobular carcinoma, SISH, 400x; B1 and C1 – same lymph node metastasis, H&E 200x; B2 – Non-amplified area in lymph node metastasis; C2 – Amplified area in lymph node metastasis, SISH 400x.



Additionally, in the primary IC cases, we identified two cases associated with non-amplified DCIS (Case 11 and 12 - table 5 and S4). Case 12 was classified as equivocal by IHC (score of 2+) because it showed strong and complete membranous staining in small groups of tumor cells, along with scattered single cells, representing less than 10% of the total tumor cell population in the sample. After SISH analysis, the same component presented HER2 gene amplification, consequently being classified as HER2 ISH negative (Figure 5).

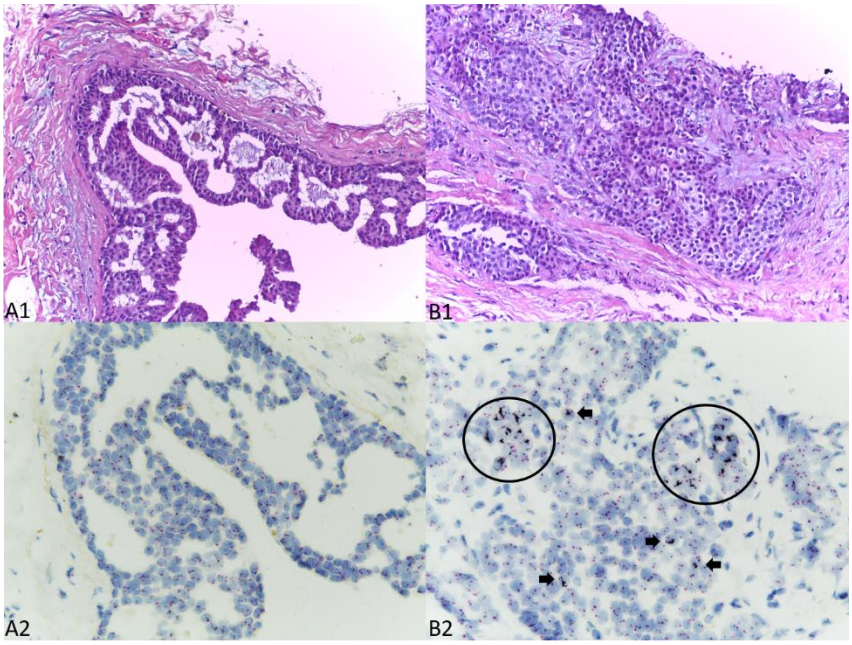
Table 5 Cases with HER2 genetic heterogeneity in invasive carcinoma associated with carcinoma in situ

Case	Gender	Age	Procedure	IC	Histologic grade	HER2 status	Proportion	CIS	Nuclear grade	HER2 status
11	F	52	NCB	NST	3	A NA	25% 75%	ductal	high	NA
12	M	73	NCB	NST	2	NA A	99% 1%	ductal	intermediate	NA

F: female; M: male; NCB: needle core biopsy; IC: invasive carcinoma; NST: no special type; A: amplified; NA: not amplified; CIS: carcinoma in situ

Figure 5
Invasive carcinoma with HER2 genetic heterogeneity associated with HER2-negative carcinoma in situ

Case 12 (2nd cohort): A1 – DCIS, H&E 200x; A2 – Non-amplified DCIS, SISH 400x; B1 – Invasive carcinoma, NST, H&E 200x; B2 – HER2 genetic heterogeneity in invasive carcinoma (HER2 gene amplification in small groups of tumor cells (circle areas) along with scattered single cells (arrows)).



5.5 Discussion

The aim of the present study was to compare the amplification *status* of HER2 gene between IC and CIS and search for HER2-GH in both components. Our results show that in all the cases, we observed the same HER2 *status* in both IC and adjacent CIS, according to previous studies [265,266]. Although, in this work, we only considerer cases with an equivocal HER2 result by IHC that can bias the results, these are the cases that require reflex testing making the issue of HER2-GH in ISH evaluation more important in this setting.

Bright-field ISH allows to better correlate tissue morphology and HER2 gene *status*, clearly identifying HER2-GH in IC and CIS [260]. In CIS, HER2-GH was recognized more frequently in SES, in which more tissue is available for evaluation. Interestingly, in these cases, only half of IC presented HER2 gene amplification, confirming that this amplification is not relevant for the transition from CIS to IC. In the literature, it has been documented that in HER2-positive cases, a significant increase occurs in HER2 copy number between primary BC and metastatic lesions [258]. The high correlation of HER2/CEP17 ratio and HER2 copy number between IC and CIS suggests that the described genetic instability of HER2 gene amplification is only present in metastatic stages.

In our study, HER2 gene amplification in IC was not associated with histological grade, contrary to what has been published [143,267]. Current evidence shows that histological grading of NCB can only be concordant with SES in about 75% of the cases [59,67]. Most of the discordant cases are upgraded in the SES, generally due to an underscored of the mitotic frequency on NCB [67,68]. Although our first cohort included a slight increase of grade 2 tumors compared with expected values in the literature, it might be the result of the large number of NCB, which can underestimate the histological grade and compromise the statistical relationship with HER2 gene

amplification [59]. Nevertheless, HER2 gene amplification in CIS was significantly associated with high nuclear grade, as previously documented [143,267].

Before the introduction of the first definition of HER2-GH, intratumoral heterogeneity was applied to discrete population of cells and reported as a rare event [268,269]. Recently, we also showed the presence of HER2-GH in IC, according to the updated ASCO/CAP HER2 guideline, to be extremely infrequent [255]. In the present work, we identified it in about 1% of the cases, including cases of male patients.

HER2-GH in the IC was observed more often in NCB, because most evaluations are performed by this procedure, which represent the first biological material on which the hormone receptors (HR) and HER2 markers should be first determined [75,97]. The predominant histologic type was invasive carcinomas, NST, with no cases classified as grade 1, as previously noticed [268]. Although we had no information regarding previous treatment, the fact that most IC cases with HER2-GH were found in NCB shows us that this rare event can occur in patients without previous treatment.

All primary invasive BC cases presented similar histological characteristics in the amplified and non-amplified areas, with HER2 gene amplification in a minor component, supporting the idea that most cases develop in a single tumor that acquired HER2 gene amplification during tumor progression. Even though molecular analysis has shown that cases with HER2-GH in the IC can be the result of two distinct tumors (also known as collision tumors), most cases appear to be clonally related resulting from clonal divergence from a single tumor, as previously shown [270,271]. However, it remains to be shown if the same process can occur in CIS.

In case 2 (second cohort), the HER2-GH was found in the lymph node metastasis rather than the primary tumor. This can be the result of tumor evolution in the lymph node metastasis or, eventually, the representation of an independent tumor that was not identified in the SES. Nevertheless, this case illustrates the importance of pathologists in selecting tumor areas with less differentiation and higher nuclear pleomorphism, either in the primary IC or in the lymph node metastases, which most likely are going to be HER2 amplified. Additionally, according to the 2013 ASCO/CAP guideline, the HER2 test should also be repeated if results are discordant with histopathologic findings [97].

Although it has been previously reported that HRs and HER2 conversion by IHC can occur between primary and metastatic lesions, the former is much more frequent (preferentially from HR-positivity in the primary tumors to HR-negativity in the metastasis) [258,272]. The conversion phenomenon could be explained by HER2-GH in the primary tumor, which can be more frequently found if more than one block is tested [273,274]. In this study, we were restricted to the analysis of only one block, which can underestimate the prevalence of HER2-GH. Additionally, we also not considered cases with HER2-GH by IHC (score 3+ in >10% and <100%). Interestingly, it has also been shown that patients with HER2-GH have worse outcome compared to patients with homogeneous amplified or non-amplified HER2 gene, suggesting that mixed tumors behave more aggressively [251,275].

Furthermore, it has been described that the majority of ICs with HER2-GH have non-amplified DCIS, consistent with our study, again supporting the idea that IC originates from CIS and that HER2 gene amplification can also be acquired in later stages [268]. Cases with HER2-GH in the IC associated with amplified DCIS probably represent distinct tumors, given the fact that the loss of the HER2 gene amplification is an unlikely event.

Finally, regarding case 12 (second cohort), which presented HER2 gene amplification in less than 10% of the total tumor cell population, a comment was made in the report recommending repetition of HER2 test by IHC in the SES to find and accurately quantify the HER2 positive component. Moreover, focally amplified small populations can be overlooked and IHC should be used to guide ISH analysis, searching for areas of potential amplification [97]. All cases exhibiting HER2-GH on NCB by ISH should have HER2 test repeated on the SES, according to the updated United Kingdom guidelines [257]. Additionally, clinical trials have not so far been based on the new 10% cut-off for ISH as provided by the 2013 ASCO/CAP guideline [105,276-279]. In fact, the presence of HER2 gene amplification was enough for inclusion of patients to HER2-targeted therapy, irrespective of the proportion of amplified cells. Fortunately, the rare presence of HER2-GH probably did not influence the clinical results given that any random group of cells evaluated will represent, most of the times, the whole tumor [280]. However, it remains to be demonstrated what is the minimal proportion of amplified tumor cell population that achieves clinical response to HER2-targeted therapy.

In conclusion, we show that HER2-GH is a rare event in IC and can already be present in CIS, not being an important step in the acquisition of invasive features. As far as we know, this is the first time that HER2-GH, according to the updated ASCO/CAP HER2 guideline, has been evaluated in both IC and adjacent CIS using bright-field ISH. Although intratumoral heterogeneity of HER2 gene amplification can have clinical significance, not only affecting the selection of patients but also explaining some of the variability of the response to targeted therapy, this is a rare event in breast cancer cases.

5.6 Supplementary information

Table S1 Comparison of HER2 and CEP17 signals between invasive and in situ carcinoma

	Invasive carcinoma	Carcinoma in situ	*p*
HER2/CEP17 ratio Median (minimum- maximum)	1.07 (0.68-11.62)	1.07 (0.65-11.23)	0.824
Average HER2 copy number Median (minimum- maximum)	1.62 (1.03-15.10)	1.57 (1.05-14.60)	0.923
Average CEP17 copy number Median (minimum- maximum)	1.55 (1.05-2.70)	1.53 (1.05-2.55)	0.577

Mann-Whitney U test

Table S2 Cases with HER2 *status* discordance between invasive carcinoma and carcinoma in situ

Case	IC HER2/CEP17 ratio	Average HER2 copy number	HER2 *status*	CIS HER2/CEP17 ratio	Average HER2 copy number	HER2 *status*	Proportion
1	11.62	15.10	A	11.23	14.60	A	40%
				0.94	1.65	NA	60%
2	2.73	3.00	A	2.20	2.75	A	60%
				1.21	1.75	NA	40%
3	1.03	1.60	NA	0.88	1.50	NA	40%
				6.07	8.50	A	60%
4	1.04	1.45	NA	1.12	1.40	NA	70%
				3.58	4.30	A	30%

IC – invasive carcinoma; A – amplified; NA – not amplified; CIS – carcinoma in situ

Table S3 Cases with HER2 genetic heterogeneity in invasive carcinoma

Case	Gender	Age	Procedure	IC	Histologic Grade	HER2/CEP17 ratio	Average HER2 copy number	HER2 *status*	Proportion
1	F	69	NCB	NST	2	7.19	9.70	A	25%
						1.14	1.65	NA	75%
2	F	45	SES	Lobular	2	5.24	8.90	A	40%
						1.00	1.40	NA	60%
3	F	60	NCB	NST	2	4.03	6.65	A	15%
						1.17	1.70	NA	85%
4	F	55	NCB	NST	2	9.17	11.00	A	15%
						1.10	1.15	NA	85%
5	F	74	SES	NST	2	2.06	3.40	A	15%
						1.21	2.00	NA	85%
6	F	55	NCB	NST	3	4.88	6.10	A	10%
						1.07	1.60	NA	90%
7	F	54	NCB	NST	3	3.63	5.45	A	10%
						1.07	2.20	NA	90%
8	F	72	NCB	NST	2	2.71	2.85	A	50%
						1.10	1.15	NA	50%
9	F	42	NCB	NST	3	7.20	7.20	A	10%
						1.11	2.00	NA	90%
10	M	65	NCB	NST	2	3.53	6.00	A	30%
						1.16	1.85	NA	70%
11	F	52	NCB	NST	3	4.50	5.40	A	25%
						1.52	2.20	NA	75%
12	M	73	NCB	NST	2	1.31	1.70	NA	99%
						7.14	10.00	A	1%

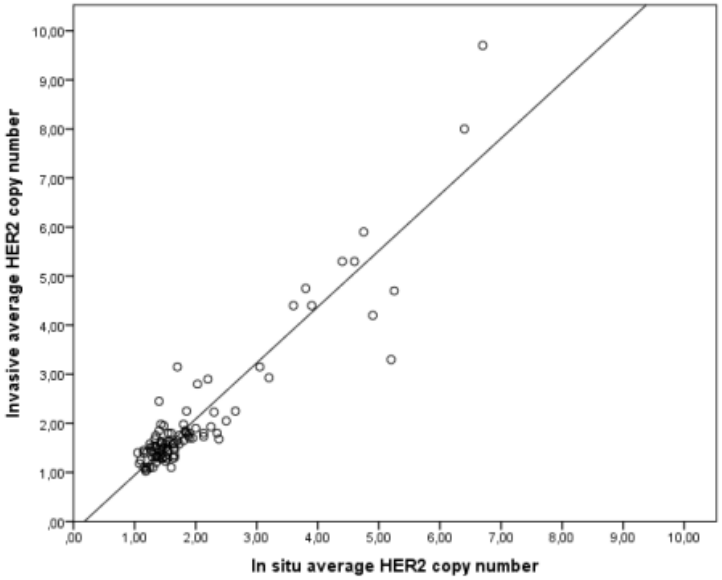
F – female; M – male; NCB – needle core biopsy; SES – surgical excision specimen; IC – invasive carcinoma; NST – no special type; A – amplified; NA – not amplified

Table S4 Cases with HER2 genetic heterogeneity in invasive carcinoma associated with carcinoma in situ

Case	IC	HER2/CEP17 ratio	Average HER2 copy number	HER2 *status*	Proportion	CIS	HER2/CEP17 ratio	Average HER2 copy number	HER2 *status*
11		4.50	5.40	A	25%				
		1.52	2.20	NA	75%		1.03	1.55	NA
12		1.31	1.70	NA	99%		0.94	1.55	NA
		7.14	10.00	A	1%				

IC – invasive carcinoma; A – amplified; NA – not amplified; CIS – carcinoma in situ

Figure S1 Relationship between average HER2 copy number *per* cell of in situ and invasive carcinoma



Chapter 6

Prognostic value of stromal TILs and PDL1 expression in breast cancer

(Polonia A et al. *J Clin Pathol.* 2017;70(10):860-867)

6.1 Abstract

Aim The present work aims to evaluate the presence of stromal tumor-infiltrating lymphocytes (TILs) and programmed cell death-ligand 1 (PDL1) expression in breast carcinomas and their correlation with available clinicopathological features.

Methods Two independent series of invasive breast cancer (IBC), one including ductal carcinoma *in situ* (DCIS) pair-matched cases, were selected, and quantification of TILs was accomplished in each case. Immunohistochemistry was also performed to evaluate the expression of PDL1.

Results In both cohorts evaluated, increased stromal TILs and PDL1 expression were present in about 10% of IBCs, being significantly associated with each other and both with grade 3 and triple-negative subtype. We observed a similar distribution of stromal TILs and PDL1 expression between DCIS and IBC. Finally, we observed that increased stromal TILs and PDL1 expression were significantly associated with cancer stem cell (CSC) markers, basal cell markers and vimentin expression. Interestingly, in IBC cases with vimentin expression, increased stromal TILs, as well as decreased PDL1 expression, disclosed a better clinical outcome, independently of the main classical BC prognostic factors.

Conclusions We have confirmed the association of stromal TILs and PDL1 expression with aggressive forms of BC and that both are already found in in situ stages. We also showed that stromal TILs and PDL1 expression are associated with clinical outcome in cases enriched for a mesenchymal immunophenotype. We describe for the first time a close relationship between CSC markers and PDL1 expression.

6.2 Introduction

In the western world, breast cancer (BC) is the most frequently diagnosed malignancy among women, representing about one-third of all new cancer cases and the second leading cause of cancer death after lung cancer.[281] BC development and progression is dependent on a complex system of different factors, including genetic and epigenetic alterations, and on factors from the tumor microenvironment, such as stromal and immune cells.[10] In fact, in recent years, numerous studies have focused on the presence and function of the host immune system and its relationship with tumor progression in a variety of solid tumors, including BC, showing that spontaneous intratumoral lymphocytic infiltrate is related to patient prognosis.[168-174]

Although the BC's inflammatory infiltrate has been studied for several decades with conflicting results, large cohorts have recently shown an association between the presence of tumor-infiltrating lymphocytes (TILs) with improved prognosis and better response to neoadjuvant chemotherapy, regardless of the absence of information of its specific immune cells.[185,186] In triple-negative (TN) BC, for instance, the presence of stromal TILs in tumor tissue at diagnosis associates with better patient outcome after adjuvant anthracycline-based chemotherapy.[187] Similarly, in human epidermal growth factor receptor 2 (HER2)-positive BC, the number of TILs in tumor tissue associates with a better response to trastuzumab treatment.[188]

However, in contrast to the presumed protective effect of TILs in tumor tissue, it has been shown that immune cells can cause the acquisition of stem cell properties by tumor cells, as well as a more pronounced mesenchymal phenotype, which are features related with a worse patient prognosis.[282] Moreover, it was also shown that tumor cells express antigens that should be recognized by patient's immune system, although most of the time the immunologic response

is unable to eliminate the cancer cells. Currently, many efforts have been made to identify molecular mechanisms that enable tumor cells to escape from the host immune system.[10] An example of tumor escape from immunosurveillance is the expression of PDL1 (programmed cell death-ligand 1) by neoplastic cells, which is a cell surface glycoprotein that conveys an inhibitory signal to T lymphocytes, through the interaction with its receptor PD1 (programmed cell death protein 1). This specific binding leads to a decrease in cytokine production and an increase of T lymphocyte apoptosis, which protect tumor cells from elimination.[196-199] Accordingly, the inhibition of this inhibitory signal by specific monoclonal antibodies, against either PDL1 or PD1, has been shown to promote tumor cell death induced by the host immune system in many cancer models.[198,209]

Based on these data, the expression of PDL1 is already being evaluated in several solid tumors, such as melanoma, non-small cell lung carcinoma and renal cell carcinoma, since it brings additional information to patient prognosis and to the selection of immunotherapy currently available targeting these molecules specifically.[200] In line with these studies, the aim of the present work was to evaluate the relationship between the presence of stromal TILs and PDL1 expression with clinicopathological features in two independent BC series. The association with disease-free survival (DFS) and overall survival (OS) was also evaluated.

6.3 Materials and methods

Tumor samples

Two independent series of BC cases were studied, both with clinical and pathological characterization performed by our group, previously described and structured in tissue microarrays (TMAs). The first cohort includes 440 primary and sporadic invasive ductal carcinomas retrieved from the Pathology Department, Hospital Xeral-Cíes, Vigo, Spain, diagnosed between 1978 and 1992, with a median age of 60.0 years (from 28 to 92 years) and a median follow-up time of 120 months (1-120 months).[283] Several parameters were extracted from the group database, including age, tumor size, histologic grade, lymph node *status*, estrogen receptor (ER), progesterone receptor (PgR), human epidermal growth factor receptor 2 (HER2) and Ki67 expression, as well as the expression of basal cell markers (CK5, CK14, epidermal growth factor receptor (EGFR) and P-cadherin), cancer stem cell (CSC) markers (CD24, CD44, CD49f and ALDH1) and the epithelial-mesenchymal transition (EMT) markers E-cadherin and vimentin. Data concerning molecular subtype, DFS and OS was also available. The main features are detailed in supplementary table S1.

The second cohort includes 94 primary in situ and invasive BC (IBC) cases, including 32 pair-matched cases, collected from the Pathology Institute of Araçatuba, São Paulo, Brazil, diagnosed between 1996 and 2006, with a median age of 55.0 years (from 32 to 96 years).[143] The data retrieved from the database include age, histological grade, lymph node *status*, hormone receptors (ER and PgR), HER2, Ki67 and molecular subtype. The main features are detailed in supplementary table S2.

This study has been performed in accordance with the national regulative law for the handling of biological specimens from tumor banks, the samples being exclusively available for research purposes in retrospective studies, as well as under the international Helsinki declaration.

Quantification of Tumor-Infiltrating Lymphocytes (TILs)

Histopathological analysis of the lymphocytic infiltrate was performed according to the guidelines for clinical and research practice.[284] Briefly, mononuclear cells, including lymphocytes and plasma cells (granulocytes excluded), were quantified in the stromal compartment as a continuous variable of 10% increment, within the borders of the invasive tumor, using visual assessment of H&E-stained sections. Thresholds were then used to categorize the continuous variable (absent - absence of TILs; slight - TILs up to 30%; moderate - TILs between 30% and 60%; marked - TILs in more than 60%). In ductal carcinoma in situ (DCIS), the lymphocytic infiltrate was quantified around the lesion using the same classification.

PDL1 immunohistochemistry

Immunohistochemical staining for PDL1 was performed in 2-3µm sections from TMAs, using a rabbit monoclonal antibody (clone SP142; 1:60 dilution; Spring Bioscience, Pleasanton, California, USA). The assay was carried out on an automated immunostaining system (Ventana BenchMark XT Staining System), using the OptiView DAB IHC Detection Kit (Ventana Medical Systems, Tucson, Arizona, USA) according to manufacturer's instructions. Positive (human placenta) and negative staining controls were performed in parallel with paraffin sections. Positivity was defined as membranous and cytoplasmic staining ≥1% in both tumor cells and stromal TILs.[285]

Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS), V.21.0, for Windows. Pearson´s chi-squared (χ^2) test (or Fisher´s exact test, when appropriate) was used for comparison of qualitative variables, and the *t* test or the Mann-Whitney U (MWU) test for quantitative variables.

Agreement rates between in situ and invasive pair-matched cases regarding presence of stromal TILs and PDL1 expression were evaluated with *k* statistics.

Survival rate curves were calculated according to the Kaplan-Meier method and compared by the log-rank test. DFS time was defined as the interval between diagnosis and BC recurrence or metastasis, whereas OS time was defined as the interval between diagnosis and BC-related death or between diagnosis and the last follow-up time for surviving patients. Multivariate survival analyses were based on the Cox proportional hazard regression model. The level of significance was set at $p < 0.05$.

6.4 Results

Association of stromal TILs and PDL1 expression with clinicopathological characteristics

The quantification of stromal TILs in the first cohort ranged between 0% and 80% (median of 10%), with a minority of cases having more than 30% (moderate to marked - 9.2%) (table 1 and figure 1). However, the presence of moderate to marked stromal TILs was significantly increased in G3 (13.6%; *p<0.001*), in ER-negative cases (16.2%; *p<0.001*), in TNBC subtype (19.0%; *p<0.001*) and in cases with high expression of Ki67 (20.0%; *p=0.019*) (table 2). A significant association between moderate to marked stromal TILs with all the evaluated basal cell markers was also observed (CK5 – 22.4%; *p<0.001* / CK14 – 28.6%; *p=0.005* / EGFR – 25.0%; *p=0.021* / P-cadherin – *15.1%; p=0.007*) (table 3). Additionally, we still found a significant association between stromal TILs and the expression of the CSC marker ALDH1 (33.3%; *p=0.014*), as well as with the expression of vimentin (17.2%; *p=0.008*) (table 3).

Table 1 Stromal TILs and PDL1 expression in BC cases from the first cohort

TILs	
Absent / Slight	178 (41.0%) / 216 (49.8%)
Moderate / Marked	36 (8.3%) / 4 (0.9%)
PDL1 expression	
Negative	407 (93.6%)
Positive	28 (6.4%)

BC, breast cancer; PDL1, programmed cell death-ligand 1; TILs, tumor-infiltrating lymphocytes.

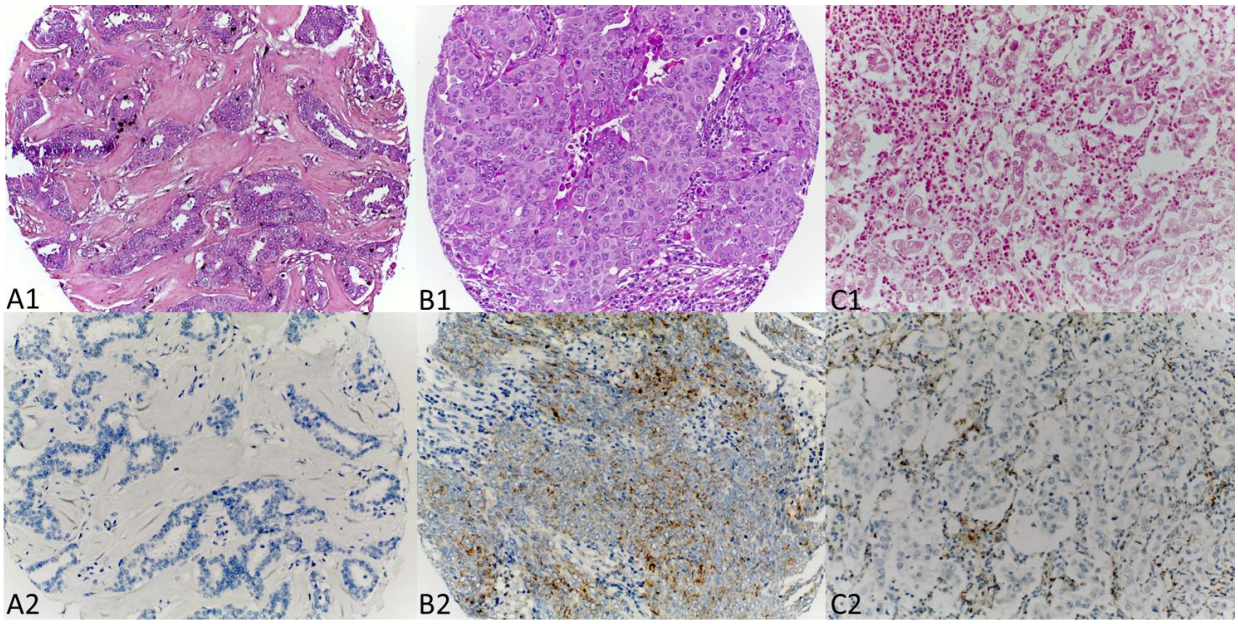


Figure 1 Stromal TILs and PDL1 expression in invasive breast cancer (200x).

A: 1 – BC without stromal TILs (H&E); 2 – PDL1 negative expression / B: 1 – BC with increased stromal TILs (H&E); 2 – PDL1 expression in cancer cells / C: 1 – BC with increased stromal TILs (H&E); 2 – PDL1 expression in stromal TILs.

Table 2 Association between clinicopathological features with stromal TILs and PDL1 expression in IBC (first cohort)

Clinicopathological features	**Stromal TILs**			**PDL1 expression**		
	absent/slight	moderate/marked	*p* value	negative	positive	*p* value
Age (average±sd)	59.40±13.11	54.88±13.48	*0.052*[a]	58.89±13.27	60.26±11.57	*0.763*[a]
Tumor size			*0.397*[b]			*0.069*[b]
≤ 2cm	87 (94.6%)	5 (5.4%)		92 (98.9%)	1 (1.1%)	
< 2 cm to ≤ 5	191 (92.3%)	16 (7.7%)		192 (92.3%)	16 (7.7%)	
> 5 cm	45 (88.2%)	6 (11.8%)		48 (92.3%)	4 (7.7%)	
Histological grade			***<0.001***[b]			***0.002***[b]
G1	71 (98.6%)	1 (1.4%)		72 (100%)	0 (0%)	
G2	105 (97.2%)	3 (2.8%)		106 (97.3%)	3 (2.7%)	
G3	171 (86.4%)	27 (13.6%)		179 (89.9%)	20 (10.1%)	
Lymph node			*0.164*[b]			*0.612*[b]
Negative	126 (88.7%)	16 (11.3%)		133 (93.0%)	10 (7.0%)	
Positive	164 (93.2%)	12 (6.8%)		168 (94.4%)	10 (5.6%)	
ER			***<0.001***[b]			***0.001***[b]
Positive	246 (95.4%)	12 (4.6%)		253 (96.9%)	8 (3.1%)	
Negative	114 (83.8%)	22 (16.2%)		120 (88.9%)	15 (11.1%)	
PgR			*0.874*[b]			*0.080*[b]
Positive	175 (91.6%)	16 (8.4%)		184 (96.3%)	7 (3.7%)	
Negative	186 (91.2%)	18 (8.8%)		190 (92.2%)	16 (7.8%)	
HER2			*0.308*[c]			*0.757*[c]
Negative	308 (91.9%)	27 (8.1%)		317 (94.6%)	18 (5.4%)	
Positive	50 (87.7%)	7 (12.3%)		55 (93.2%)	4 (6.8%)	
Ki67			***0.019***[a]			***0.002***[a]
< 14%	342 (92.2%)	29 (7.8%)		354 (94.9%)	19 (5.1%)	
≥ 14%	20 (80.0%)	5 (20.0%)		21 (84%)	4 (16.0%)	
Molecular subtype			***<0.001***[bd]			***<0.001***[cd]
Luminal A	248 (95.4%)	12 (4.6%)		253 (97.7%)	6 (2.3%)	
Luminal B	24 (85.7%)	4 (14.3%)		26 (86.7%)	4 (13.3%)	
HER2-positive	26 (89.7%)	3 (10.3%)		29 (100%)	0 (0%)	
TNBC	64 (81.0%)	15 (19.0%)		67 (83.8%)	13 (16.2%)	

Bold indicated p<0.05 is statistically significant. [a] - Mann-Whitney *U* test; [b] – Pearson´s χ^2 test; [c] – Fisher´s exact test; [d] - TN vs Others; sd – standard deviation.

Table 3 Association between basal cell markers, CSC markers and EMT markers with stromal TILs and PDL1 expression in invasive breast cancer (first cohort)

	Stromal TILs			**PDL1 expression**		
	absent/slight	moderate/marked	*p* value	negative	positive	*p* value
Basal cell markers						
CK5			***<0.001***[a]			***<0.001***[a]
Negative	317 (93.8%)	21 (6.2%)		327 (96.5%)	12 (3.5%)	
Positive	45 (77.6%)	13 (22.4%)		48 (81.4%)	11 (18.6%)	
CK14			***0.005***[a]			***0.031***[a]
Negative	347 (92.5%)	28 (7.5%)		357 (94.9%)	19 (5.1%)	
Positive	15 (71.4%)	6 (28.6%)		18 (81.8%)	4 (18.2%)	
EGFR			***0.021***[a]			***0.022***[a]
Negative	347 (92.3%)	29 (7.7%)		359 (95.0%)	19 (5.0%)	
Positive	15 (75.0%)	5 (25.0%)		16 (80.0%)	4 (20.0%)	
P-cadherin			***0.007***[b]			***<0.001***[b]
Negative	278 (93.6%)	19 (6.4%)		289 (96.7%)	10 (3.3%)	
Positive	84 (84.9%)	15 (15.1%)		86 (86.9%)	13 (13.1%)	
CSC markers						
$CD44^{+}/CD24^{-/low}$			*0.165*[b]			***0.044***[b]
Negative	204 (93.2%)	15 (6.8%)		210 (96.3%)	8 (3.7%)	
Positive	157 (89.2%)	19 (10.8%)		163 (91.6%)	15 (8.4%)	
CD49f			*0.251*[a]			***0.011***[a]
Negative	318 (91.9%)	28 (8.1%)		330 (95.4%)	16 (4.6%)	
Positive	38 (86.4%)	6 (13.6%)		39 (84.8%)	7 (15.2%)	
ALDH1			***0.014***[a]			***0.033***[a]
Negative	354 (92.2%)	30 (7.8%)		365 (94.8%)	20 (5.2%)	
Positive	8 (66.7%)	4 (33.3%)		10 (76.9%)	3 (23.1%)	
EMT markers						
E-cadherin			*0.708*[a]			*0.628*[a]
Negative/Low	21 (95.5%)	1 (4.5%)		22 (100%)	0 (0%)	
Positive	339 (91.1%)	33 (8.9%)		352 (93.9%)	23 (6.1%)	
Vimentin			***0.008***[b]			***0.036***[a]
Negative	307 (93.0%)	23 (7.0%)		316 (95.5%)	15 (4.5%)	
Positive	53 (82.8%)	11 (17.2%)		57 (87.7%)	8 (12.3%)	

Bold indicated $p<0.05$ is statistically significant. [a] – Fisher´s exact test; [b] – Pearson´s χ^2 test.

The expression of PDL1 was mainly found at the cell membrane (in both tumor cells and stromal TILs) and only present in 6.4% of the cases (table 1 and figure 1). Although PDL1 expression was not observed in normal breast tissue, it was significantly associated to G3 (10.1%; *p=0.002*) and ER-negative carcinomas (11.1%; *p=0.001*), as well as with the TNBC subtype (16.2%; *p<0.001*) and with cases with high expression of Ki67 (16.0%; *p=0.002*) (table 2). We also observed a significant direct association between PDL1 expression and all the evaluated basal cell markers (CK5 – 18.6%; *p<0.001* / CK14 – 18.2%; *p=0.031* / EGFR – 20.0%; *p=0.022* / P-cadherin – 13.1%; *p<0.001*), all the evaluated CSC markers ($CD44^{+}/CD24^{-/low}$ - 8.4%; *p=0.044* / CD49f – 15.2%; *p=0.011* / ALDH1 - 23.1%; *p=0.033*) and with the expression of vimentin (12.3%; *p=0.036*) (table 3).

Stromal TILs and PDL1 expression were still significantly associated between each other (64.3%; *p<0.001*) (table 4).

Table 4 Association between stromal TILs and PDL1 expression in invasive breast cancer (first cohort)

	Stromal TILs			**PDL1 expression**		
	absent/slight	moderate/marked	*p* value	negative	positive	*p* value
PDL1 expression			***<0.001***[a]			na
Negative	380 (94.8%)	21 (5.2%)		-	-	
Positive	10 (35.7%)	18 (64.3%)		-	-	
DFS (mean of months)	86.5	97.8	*0.087*[b]	87.5	92.3	*0.327*[b]
OS (mean of months)	91.7	99.8	*0.108*[b]	92.7	92.9	*0.568*[b]

Bold indicated p<0.05 is statistically significant. [a] – Fisher´s exact test; [b] - Kaplan-Meier method/log-rank *p* value; na, not applicable; DFS, disease-free survival; OS, overall survival.

Association of stromal TILs and PDL1 expression with patient prognosis

Overall, neither stromal TILs nor PDL1 expression were significantly associated with DFS or OS rates (table 4). However, in both G3 and ER-negative cases, the presence of more than 30% of stromal TILs was significantly associated with better DFS rates (94.26 vs 76.36; *p=0.045* and 93.01 vs 68.02; *p=0.044*, respectively). Additionally, also in G3 BC cases, PDL1 expression was significantly associated with improved DFS (96.79 vs 77.39; *p=0.043*). In vimentin-positive BC cases, increased stromal TILs were also significantly associated with better DFS and OS rates (110.18 vs 77.35; *p=0.037* and 113.36 vs 85.39; *p=0.047*, respectively) (supplementary table S3).

However, in multivariate analysis, only increased stromal TILs in vimentin-positive BC were independently associated with better DFS and OS (HR=0.10, *p=0.017* and HR=0.06, *p=0.019*, respectively). Interestingly, also within vimentin-positive cases, PDL1 expression was, in contrast, significantly associated with a decreased OS (HR=7.12, *p=0.018*) (supplementary table S4 and figure 2).

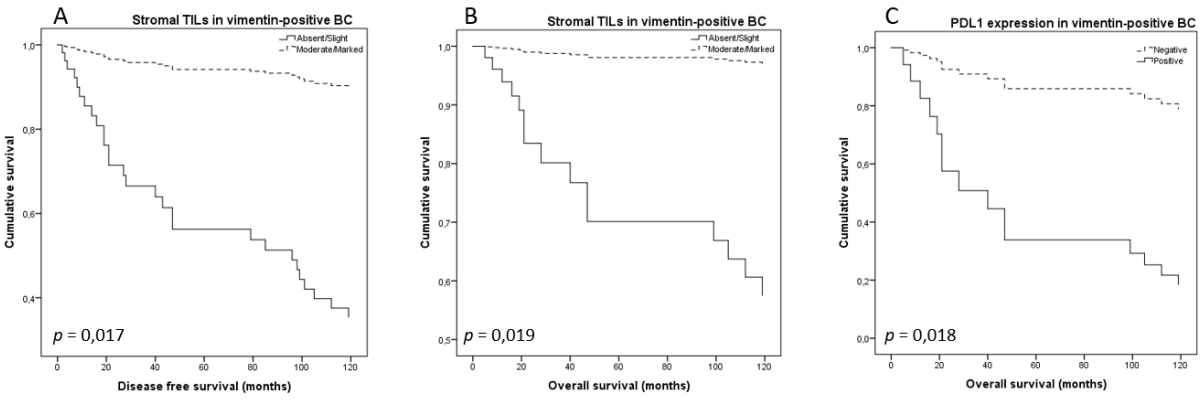


Figure 2 Survival curves showing the significant DFS and OS for patients with vimentin-positive BC relatively to stromal TILs (A and B) and PDL1 expression (C).

Concerning molecular subtypes, we observed that moderate to marked stromal TILs were associated with better DFS (100.4 vs 72.6) and OS (103.8 vs 79.9) rates (*p=0.096* and *p=0.080*, respectively) in TNBC, although without reaching statistical significance (supplementary table S5).

Stromal TILs and PDL1 expression in matched in situ and invasive carcinomas

When stromal TILs and PDL1 expression were compared between DCIS and IBC, a similar distribution was found (TILs – 13.0% vs 13.8%; *p=0.911*; PDL1 expression - 8.7% vs 13.8%; *p=0.399*, respectively), as actually happened for all the other characteristics previously evaluated (figure 3, tables 5 and supplementary S2).

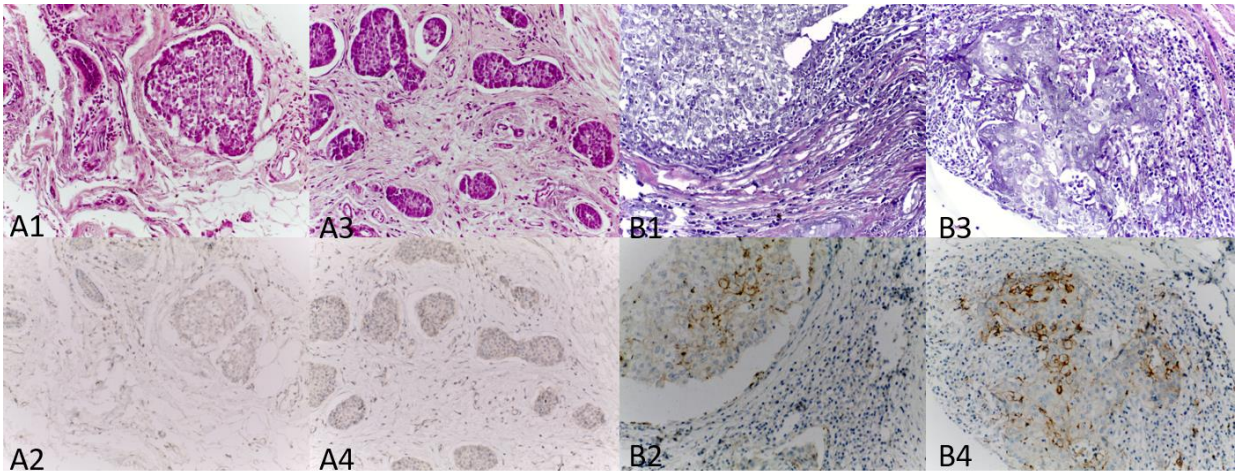


Figure 3 Stromal TILs and PDL1 expression in pair-matched in situ and invasive breast cancer (200x).
A: 1 – DCIS without stromal TILs (H&E); 2 – PDL1-negative expression in DCIS; 3 – IBC with few stromal TILs (H&E); 4 – PDL1-negative expression in IBC / B: 1 – DCIS with increased stromal TILs (H&E); 2 – PDL1 expression in DCIS; 3 – IBC with increased stromal TILs (H&E); 3 – PDL1 expression in IBC.

Table 5 Tumor-infiltrating lymphocytes and PDL1 expression in breast cancer cases from the second cohort

	DCIS	**Invasive carcinoma**	*p* value
Tumor-infiltrating lymphocytes			*0.911*[a]
Absent/Slight	40 (87.0%)	69 (86.2%)	
Moderate/Marked	6 (13.0%)	11 (13.8%)	
PDL1 expression			*0.399*[a]
Negative	42 (91.3%)	69 (86.2%)	
Positive	4 (8.7%)	11 (13.8%)	

[a] – Pearson´s χ^2 test. DCIS, ductal carcinoma in situ.

Validating the results found in the first cohort, the presence of moderate to marked stromal TILs was significantly increased in G3 (26.9%; *p=0.001*), in TNBC subtype (25%; *p=0.023*) and in cases with PDL1 expression (63.6%; *p=<0.001*) (supplementary table S6). We also observed an increase of stromal TILs in ER-negative cases and in cases with high expression of Ki67 (20%; *p=0.059* and 20%; *p=0.057*, respectively). Additionally, we found a similar association of increased stromal TILs and HER2-positive subtype (25%; *p=0.023*). In DCIS, moderate to marked stromal TILs were enriched in G3 (23.5%; *p=0.071*), TNBC and HER2-positive subtypes (20% and 40%, respectively; *p=0.073*) and in cases with PDL1 expression (50%; *p=0.077*), although without reaching statistical significance.

Concerning PDL1 expression, it was significantly associated with G3 (26.9%; *p=0.005*) and all molecular subtypes, except luminal A (0%; *p=0.005*) (supplementary table S7). Interestingly, we also observed PDL1 expression enriched in cases with high expression of Ki67 (30%; *p=0.061*), without reaching statistical significance.

Finally, regarding the pair-matched cases, we observed a concordance rate of stromal TILs and PDL1 expression between DCIS and invasive BC of 93.8% (30/32) and 81.3% (26/32),

respectively (table 6 and 7). Furthermore, in PDL1 expression, we found that 5 out 6 discordant cases changed from negative to positive expression between DCIS and IBC, respectively.

Table 6 Concordance of stromal TILs between DCIS and invasive carcinoma

Stromal TILs	**Invasive carcinoma**		
	absent/slight	moderate/marked	Total
DCIS			
absent/slight	27	2	29
moderate/marked	0	3	3
Total	27	5	32

Kappa statistics – 0.72; *p*=<**0.001**. Bold indicated p<0.05 is statistically significant. DCIS, ductal carcinoma in situ.

Table 7 Concordance of PDL1 expression between DCIS and invasive carcinoma

PDL1	**Invasive carcinoma**		
	Negative	Positive	Total
DCIS			
Negative	24	5	29
Positive	1	2	3
Total	25	7	32

Kappa statistics – 0.31; *p*=**0.049**. Bold indicated p<0.05 is statistically significant. DCIS, ductal carcinoma in situ.

6.5 Discussion

Our study confirms that increased stromal TILs are present in a minority of IBC cases and that are already present in in situ stages, as previously shown.[185,286] Although PDL1 expression has been reported in the literature from 20% to nearly 60% of the cases, we only observed in a small proportion of IBCs and, for the first time, even in DCIS cells.[206,207,287-289]

In IBC, increased stromal TILs and PDL1 expression were associated with each other and with G3 and TNBC subtype, with statistical significance in both cohorts, which also validates previous reports.[207,286,288] The association of increased stromal TILs and PDL1 expression with the evaluated basal cell markers also reinforces the relationship with the TNBC molecular subtype.

Stromal TILs include several types of T and B lymphoid cells in different proportions with distinct associations with clinical outcome that we did not discriminate in our study. For instance, cytotoxic (CD8+) T cells are significantly increased in high grade, ER-negative BC and BC with increased proliferative activity, as well as associated with improved clinical outcome.[178-180] On the other side, immunosuppressive (CD4+/FOXP3+) T cells have been shown to be associated with worse prognosis in IBC and even with increased risk of relapse in DCIS.[181-183] This data suggest that cytotoxic T cells are responsible for the antitumor immune activity and immunosuppressive T cells can inhibit this response. Furthermore, B lymphocytes are also associated with higher histological grade, ER-negative cases and basal phenotype, as well as with better prognosis.[184] This indicates that humoral immune response, along with cell-mediated immune response, acts in convergence to achieve effective antitumor response. Based on this knowledge, it would be important to evaluate the clinical value of subtyping the composition of stromal TILs in BC and reveal the potential predictive role of these markers in response to

immunotherapy. Nonetheless, it is remarkable that the quantification of stromal TILs alone, regardless of its specific subpopulation of lymphoid cells, has prognostic and predictive information.

Although the use of TMAs in this work could, eventually, miss quantify stromal TILs evaluation, several reports suggest that TMAs are a valid choice, as the majority of BC are not heterogeneous regarding stromal TILs distribution throughout the tumor.[179,284,287,290] While recent guidelines recommend the evaluation of TILs as a continuous variable, we were able to show that the cut-off values used in this work enclose clinical significance and may be used to easily categorize this parameter, possibly reducing the interobserver variability.

Concerning PDL1 expression, in addition to tumor cells, it has been reported that it can be also present in stromal TILs, as observed by us, specifically in CD4+/FOXP3- T cells.[288] However, PDL1 expression is not associated with T cell exhaustion markers, which means that PDL1 is only partially inhibiting T cells.[207] Nevertheless, this does not contradict the idea of blocking PDL1 in order to reactivate partially inhibited T cells and further increase the antitumor immune response.

The monoclonal antibody that has been used in this work was the clone SP142, which was developed for anti-PDL1 immunotherapy (atezolizumab, formerly MPDL3280A).[200,285,291] SP142 is specific for the PDL1 cytoplasmic domain giving clear membranous staining, with lower background staining.[292] The different results of PDL1 expression in BC are probably related to different methods in evaluating PDL1 expression, from protein expression (using immunohistochemistry or immunofluorescence) to mRNA analysis (using PCR, in situ hybridization or microarrays), different cut-offs values, antibodies used and constitution of cohorts, as well as heterogeneity within tumors.[216] Currently, the assays to evaluate the role of

PDL1 expression for prognosis and predictive information are not standardized neither in methodology nor interpretation of the staining. Consequently, it is of major importance that the clinical significance of PDL1 expression in BC is revealed before its use in daily routine.

Concerning DCIS, a similar trend in association between stromal TILs and PDL1 expression with histological grade and molecular subtype was also detected, suggesting that, as we previously reported, these features correlate well with the invasive counterpart.[143] Very recently, the expression of PDL1 has been described in about 80% of stromal TILs associated with DCIS cases, as well as an association with TNBC subtype.[293] In that work, there was no expression of PDL1 in DCIS cells, unlike our observation, in which we detected in both DCIS cells and associated stromal TILs.

The above-mentioned findings in DCIS samples indicate that the immune response to tumors exists even at early stages and that distinct molecular subtypes have different immunogenicity. The characterization of immune checkpoint markers, in both invasive and DCIS cases, may result in potential targets for therapy, such as anti-PDL1 treatment. Moreover, we also report a high concordance of stromal TILs and PDL1 expression between DCIS and IBC pair-matched cases. This means that stromal TILs and PDL1 expression might not be relevant for the progression from in situ to IBC, but instead represent a response to intrinsic characteristics of different molecular subtypes. Nevertheless, although the few discordant cases might be due to sampling issues, the fact that 5 out of 6 cases changed PDL1 expression from negative to positive between in situ and invasive BC, respectively, could also mean that an additional activation of the PDL1 pathway can be achieved in BC progression. As such, it remains to be clarified the complex role of the immune response in the progression from in situ to invasive disease.

Finally, we observed that stromal TILs and PDL1 expression were associated with CSC markers and vimentin expression. Our results are expected because stromal TILs and PDL1 expression are associated with TNBC subtype, which in turn is associated with a higher proportion of cells with stem cell phenotype ($CD44^{+}/CD24^{-/low}$, CD49f and ALDH1) and EMT features, as shown by our group in the past.[120,283,294] In addition, as stated above, both stromal TILs and PDL1 expression were associated with basal cell markers, including P-cadherin, which was previously demonstrated by our group to be up-regulated in basal–like subtype and associated with stem cell properties and EMT phenotype in BC cell lines.[120,294-296]

Although the association of stromal TILs with stem cell phenotype and EMT has been previously reported, as far as we know, this is the first time that PDL1 expression in BC cases is associated with CSC markers ($CD44^{+}/CD24^{-/low}$, CD49f and ALDH1), which is probably related to the close relationship with stromal TILs and TNBC subtype.[178] However, we observed an association of PDL1 expression with all CSC markers, while stromal TILs were only associated with ALDH1. This probably means a potential role of CSCs in the activation of PDL1 expression, both in tumor cells and stromal TILs, directly regulating the antitumoral immune response.

Equally, PDL1 expression has been characterized recently in relation with EMT markers.[297] The authors showed that the induction of EMT causes the up-regulation of PDL1 expression in BC cell lines and that the inhibition of PDL1 originates an EMT reversal state, suggesting a dual benefit of inhibiting the PDL1 pathway. They also showed an association of PDL1 expression with EMT markers in BC patients, such as vimentin, as our study.

Despite the association of stromal TILs with CSC and EMT markers, the final effect in BC tissue is a protective one, given the fact that, in vimentin-positive BC, the presence of more

than 30% of stromal TILs were independently associated with increased DFS and OS rates, reflecting an active antitumor immune response with an impact on patient outcome. Although contradictory information has been published regarding PDL1 expression and BC prognosis, in theory, the expression of PDL1 in BC should have a deleterious effect on prognosis, given the inhibitory effect of PDL1 in the antitumor immune response.[206] We also showed that in vimentin-positive BC, PDL1 expression is independently associated with worse OS. Although it has been reported that PDL1 expression can be related with better survival rates in BC, it is probably due to the close association of PDL1 with the presence of increased stromal TILs, the true effectors against tumor cells.[207,208] Finally, because IBC with mesenchymal traits is strongly associated with TNBC, a subtype associated with worse prognosis and limited treatment strategies, immunologic modulators may represent an alternative approach to improve clinical outcome of these patients.[186,206,298]

In conclusion, we have confirmed the association of stromal TILs and PDL1 expression with aggressive BC (G3 and TNBC/basal subtype) and that both are already expressed in in situ stages. We also showed that increased stromal TILs and PDL1 expression are associated with clinical outcome in BC with vimentin expression. Notably, we describe a close relationship between CSC markers with stromal TILs, and for the first time, with PDL1 expression.

6.6 Supplementary information

Table S1 Clinicopathological features of the first cohort

Age (average ± sd)	59.02 ± 13.14
Tumor size	
≤ 2cm	94 (26.4%)
< 2cm to ≤ 5cm	210 (59.0%)
> 5cm	52 (14.6%)
Histological grade	
G1	73 (19.0%)
G2	110 (28.7%)
G3	201 (52.3%)
Lymph node status	
Negative	144 (44.7%)
Positive	178 (55.3%)
ER	
Positive	263 (65.8%)
Negative	137 (34.2%)
PgR	
Positive	194 (48.4%)
Negative	207 (51.6%)
HER2	
Negative	339 (85.2%)
Positive	59 (14.8%)
Ki67	
< 14%	377 (93.8%)
≥ 14%	25 (6.2%)
Molecular subtype	
Luminal A	263 (65.4%)
Luminal B	30 (7.5%)
HER2-positive	29 (7.2%)
TNBC	80 (19.9%)

sd – standard deviation

Table S1 Clinicopathological features of the first cohort (cont.)

Basal cell markers		
CK5		
	Negative	343 (85.3%)
	Positive	59 (14.7%)
CK14		
	Negative	380 (94.5%)
	Positive	22 (5.5%)
EGFR		
	Negative	382 (95.0%)
	Positive	20 (5.0%)
P-cadherin		
	Negative	302 (75.1%)
	Positive	100 (24.9%)
CSC markers		
$CD44^{+}/CD24^{-/low}$		
	Negative	221 (55.3%)
	Positive	179 (44.7%)
CD49f		
	Negative	348 (88.3%)
	Positive	46 (11.7%)
ALDH1		
	Negative	389 (96.8%)
	Positive	13 (3.2%)
EMT markers		
E-cadherin		
	Negative/Low	22 (5.5%)
	Positive	378 (94.5%)
Vimentin		
	Negative	335 (83.8%)
	Positive	65 (16.2%)

Table S2 Clinicopathological features of the second cohort

	DCIS	**Invasive carcinoma**	*p*
Age (average ± sd)	55.65 ± 13.18	55.65 ± 13.18	na
Histological grade			*0.566*[a]
G1	21 (26.6%)	27 (34.2%)	
G2	27 (34.2%)	23 (29.1%)	
G3	31 (39.2%)	29 (36.7%)	
Lymph node status			na
Negative	-	16 (28.1%)	
Positive	-	41 (71.9%)	
ER			*0.431*[b]
Positive	53 (67.1%)	51 (64.6%)	
Negative	26 (32.9%)	28 (35.4%)	
PgR			*0.693*[b]
Positive	35 (44.9%)	34 (43%)	
Negative	43 (55.1%)	45 (57%)	
HER2			*1.000*[a]
Negative	58 (73.4%)	58 (73.4%)	
Positive	21 (26.6%)	21 (26.6%)	
Ki67			*0.555*[b]
< 14%	69 (88.5%)	67 (85.9%)	
≥ 14%	9 (11.5%)	11 (14.1%)	
Molecular subtype			*0.901*[a]
Luminal A	45 (57.0%)	41 (52.0%)	
Luminal B	12 (15.2%)	15 (18.9%)	
HER2-positive	14 (17.7%)	14 (17.7%)	
TNBC	8 (10.1%)	9 (11.4%)	

sd – standard deviation; na – not applicable; [a] – Pearson´s χ^2 test; [b] – Mann-Whitney *U* test

Table S3 Relationship between stromal TILs and PDL1 expression with DFS and OS in different subgroups

Subgroup	**Stromal TILs**			**PDL1 expression**		
	absent/slight	moderate/marked	*p*	negative	positive	*p*
G3						
DFS	76.36	94.26	***0.045***	77.39	96.79	***0.043***
OS	82.98	96.62	*0.062*	83.99	97.47	*0.100*
ER negative						
DFS	68.02	93.01	***0.044***	69.59	90.60	*0.097*
OS	75.02	95.97	*0.056*	76.64	91.47	*0.201*
Vimentin positive						
DFS	77.35	110.18	***0.037***	83.91	84.38	*0.618*
OS	85.39	113.36	***0.047***	91.91	85.00	*0.926*

DFS and OS: mean of months. Bold indicated $p<0.05$ is statistically significant.

Table S4 Multivariate Cox proportional hazard analysis

Variable	Evaluation	G3 DFS		ER negative DFS		Vimentin positive DFS		OS	
		HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*
Stromal TILs	absent/slight	1		1		1		1	
	moderate/marked	0.89 (0.37-2.17)	*0.800*	0.66 (0.27-1.61)	*0.365*	0.10 (0.02-0.67)	***0.017***	0.06 (0.01-0.62)	***0.019***
PDL1	Negative	1		1		1		1	
	Positive	0.60 (0.20-1.80)	*0.361*	0.58 (0.21-1.62)	*0.301*	3.20 (0.67-15.25)	*0.144*	7.12 (1.40-36.34)	***0.018***
Tumor size	≤2cm	1		1		1		1	
	<2cm to ≤5cm	1.48 (0.74-2.93)	*0.266*	1.88 (0.87-4.07)	*0.108*	1.18 (0.37-3.84)	*0.779*	1.58 (0.25-9.80)	*0.626*
	>5cm	2.16 (0.97-4.80)	*0.059*	2.45 (1.04-5.75)	***0.040***	2.96 (0.83-10.49)	*0.093*	2.25 (0.29-17.50)	*0.439*
Lymph node status	Negative	1		nc		nc		1	
	Positive	1.74 (1.06-2.87)	***0.030***					1.54 (0.48-4.98)	*0.471*
Histological grade	G1			1		1		1	
	G2	nc		1.43 (0.38-5.42)	*0.603*	1.13 (0.18-7.18)	*0.901*	2.34 (0.11-49.18)	*0.585*
	G3			1.73 (0.54-5.59)	*0.360*	3.40 (0.66-17.50)	*0.144*	1.71 (0.14-21.35)	*0.679*
ER	Positive	nc		nc		1		1	
	Negative					0.60 (0.14-2.55)	*0.493*	2.24 (0.19-26.48)	*0.523*

HR – hazard ratio; CI – confidence interval; nc – not computed. Bold indicated p<0.05 is statistically significant.

Table S5 Relationship between DFS and OS with TILs and PDL1 expression in molecular subtypes

Molecular subtype	**Stromal TILs**			**PDL1 expression**		
	absent/slight	moderate/marked	*p*	negative	positive	*p*
Luminal A						
DFS	94.8	108.6	*0.130*	-	-	nc
OS	99.1	108.6	*0.180*	-	-	nc
Luminal B						
DFS	75.5	94.3	*0.507*	84.4	58.8	*0.312*
OS	77.3	94.3	*0.507*	86.1	58.8	*0.291*
HER2-positive						
DFS	49.0	56.0	*0.769*	-	-	nc
OS	60.5	59.7	*0.978*	-	-	nc
TNBC						
DFS	72.6	100.4	*0.096*	76.1	92.0	*0.239*
OS	79.9	103.8	*0.080*	83.6	93.0	*0.417*

DFS and OS: mean of months; nc – not computed

Table S6 Association between clinicopathological features with stromal TILs in DCIS and IBC (2nd cohort)

Clinicopathological features	DCIS			Invasive carcinoma		
	Stromal TILs			Stromal TILS		
	absent/slight	moderate/marked	*p*	absent/slight	moderate/marked	*p*
Age (average ± sd)	55.82±14.77	60.00±14.93	*0.555*[a]	54.80±12.90	61.86±10.37	*0.169*[a]
Histological grade			*0.071*[b]			***0.001***[b]
G1/G2	25 (96.2%)	1 (3.8%)		40 (100%)	0 (0%)	
G3	13 (76.5%)	4 (23.5%)		19 (73.1%)	7 (26.9%)	
Lymph node status			nc			*1.000*[b]
Negative	-	-		11 (91.7%)	1 (8.3%)	
Positive	-	-		32 (86.5%)	5 (13.5%)	
ER			*0.241*[c]			*0.059*[c]
Positive	30 (93.8%)	2 (6.2%)		39 (95.1%)	2 (4.9%)	
Negative	8 (72.7%)	3 (27.3%)		20 (80%)	5 (20%)	
PgR			*0.706*[c]			*0.207*[c]
Positive	18 (90%)	2 (10%)		27 (96.4%)	1 (3.6%)	
Negative	19 (86.4%)	3 (13.6%)		32 (84.2%)	6 (15.8%)	
HER2			*0.277*[b]			*0.380*[b]
Negative	31 (91.2%)	3 (8.8%)		44 (91.7%)	4 (8.3%)	
Positive	7 (77.8%)	2 (22.2%)		15 (83.3%)	3 (16.7%)	
Ki67			*0.308*[c]			*0.057*[c]
< 14%	33 (91.7%)	3 (8.3%)		50 (90.9%)	5 (9.1%)	
≥ 14%	5 (71.4%)	2 (28.6%)		8 (80%)	2 (20%)	
Molecular subtype			*0.073*[bd]			***0.023***[bd]
Luminal A	24 (96%)	1 (4%)		32 (100%)	0 (0%)	
Luminal B	7 (87.5%)	1 (12.5%)		12 (85.7%)	2 (14.3%)	
HER2-positive	3 (60%)	2 (40%)		9 (75%)	3 (25%)	
TNBC	4 (80%)	1 (20%)		6 (75%)	2 (25%)	
PDL1 expression			*0.077*[b]			***<0.001***[b]
Negative	38 (90.5%)	4 (9.5%)		65 (94.2%)	4 (5.8%)	
Positive	2 (50%)	2 (50%)		4 (36.4%)	7 (63.6%)	

[a] – *t*-test; [b] – Fisher´s exact test; [c] - Mann-Whitney *U* test; [d] - Luminal A/B vs HER2-positive/TNBC; sd – standard deviation; nc – not computed. Bold indicated p<0.05 is statistically significant.

Table S7 Association between clinicopathological features with PDL1 expression in DCIS and IBC (2nd cohort)

Clinicopathological features	**DCIS**			**Invasive carcinoma**		
	PDL1			**PDL1**		
	negative	positive	*p*	negative	positive	*p*
Age (average ± sd)	57.26±14.38	47.00±16.27	*0.186*[a]	55.17±12.95	58.25±11.87	*0.527*[a]
Histological grade			*1.000*[b]			***0.005***[b]
G1/G2	24 (92.3%)	2 (7.7%)		39 (97.5%)	1 (2.5%)	
G3	15 (88.2%)	2 (11.8%)		19 (73.1%)	7 (26.9%)	
Lymph node			nc			*0.340*[b]
Negative	-	-		9 (75%)	3 (25%)	
Positive	-	-		33 (89.2%)	4 (10.8%)	
ER			*0.531*[c]			*0.185*[c]
Positive	30 (93.8%)	2 (6.2%)		38 (92.7%)	3 (7.3%)	
Negative	9 (81.8%)	2 (18.2%)		20 (80%)	5 (20%)	
PgR			*0.951*[c]			*0.878*[c]
Positive	18 (90%)	2 (10%)		25 (89.3%)	3 (10.7%)	
Negative	20 (90.9%)	2 (9.1%)		33 (86.8%)	5 (13.2%)	
HER2			*1.000*[b]			*0.673*[b]
Negative	31 (91.2%)	3 (8.8%)		43 (89.6%)	5 (10.4%)	
Positive	8 (88.9%)	1 (11.1%)		15 (83.3%)	3 (16.7%)	
Ki67			*0.531*[c]			*0.061*[c]
< 14%	32 (88.9%)	4 (11.1%)		50 (90.9%)	5 (9.1%)	
≥ 14%	7 (100%)	0 (0%)		7 (70%)	3 (30%)	
Molecular subtype			*0.226*[bd]			***0.005***[be]
Luminal A	23 (92%)	2 (8%)		32 (100%)	0 (0%)	
Luminal B	8 (100%)	0 (0%)		10 (71.4%)	4 (28.6%)	
HER2-positive	4 (80%)	1 (20%)		10 (83.3%)	2 (16.7%)	
TNBC	4 (80%)	1 (20%)		6 (75%)	2 (25%)	

[a] – *t*-test; [b] – Fisher´s exact test; [c] - Mann-Whitney *U* test; [d] - Luminal A/B vs HER2-positive/TNBC; [e] – Luminal A vs Others; sd – standard deviation; nc – not computed. Bold indicated p<0.05 is statistically significant.

Chapter 7

General Discussion

BC is the most frequent type of cancer in women representing about one-third of all new diagnosis.[27] Portugal is not an exception to this rule and BC is the main cause of cancer-related death in women.[29] As such, BC emerges as one of the most important models to be studied if we want to contribute to impact on cancer in general.

Given that survival rates decrease rapidly from localized to metastatic stages, the early detection of this cancer, as well as all others, is the most key factor for the success of its treatment.[27] Other crucial factors that should be taken into consideration, if we aim to decrease BC incidence, is the knowledge concerning risk factors. The proper identification of such risk factors can enable the modification of lifestyle habits to decrease the probability of developing BC.[38-41] Nevertheless, although environment factors play a very relevant role in the etiology of BC, most of our knowledge is related to luminal BC (estrogen receptor positive), leaving triple-negative and HER2-positive BCs, which represent more aggressive forms of BC, outside of this possible intervention.[42]

Molecular classification of BC has transformed our understanding about this disease and the application of this methodology into different models has revolutionized cancer classification. Even though gene expression profiling has been available for more than 15 years, the molecular classification is not a current practice, being applied to very specific cases with very specific questions in mind.[94] On the other side, old markers such as histologic classification and grading are a mandatory requirement in any BC pathology report, being important prognostic factors correlating with disease-free and overall survival.[61]

Finally, IHC studies finish the evaluation of BC cases, providing valuable information regarding prognosis and, more importantly, prediction to targeted-therapy.[98-100,103-106] Additionally, IHC can even be used to classify BC cases into molecular subtypes, which has

been shown to be clinically useful.[60] The application of these markers, as well as others that complement the pathology report, try to provide the most accurate and personalized classification and stratification of BC patients. Nevertheless, patients with the same classification and stratification present different outcomes. For these reasons, improving prognostication in BC, through the refinement of current biomarkers and identification of new ones, remains an important task to delineate the precise risk of BC patients and provide them with personalized treatment.

As such, in this thesis, we studied three biomarkers, starting with the oncogene HER2 to improve its value in the identification of patients to targeted therapy. The first work aimed to understand the impact of the new ASCO/CAP guideline for HER2 test in BC. The 2013 ASCO/CAP guideline came to replace the previous 2007 guideline and presented a double change: modification of IHC criteria, that changes the classification of which cases are selected to reflex testing, and modification of ISH criteria, that changes the classification of which cases are positive or negative for HER2 gene amplification. For this purpose, we collected cases that were classified before and after the introduction of the new ASCO/CAP guideline. The different classification of the cases reveals that the 2013 ASCO/CAP guideline has a relevant impact on the classification of BC cases (significantly increasing the number of HER2-positive cases), although not revealing if the change was due to the modification of IHC criteria, ISH criteria or both. To answer this question, we compared the cases from before and after the new guideline classifying with the same ISH criteria, allowing the determination of the value of IHC in the impact of the new guideline. The absence of different results in this setting reveals that the modification of IHC criteria had negligible effect on the final change of the new guideline, as expected.[238] On the other hand, the classification of the two case series with different ISH criteria

(2007 and 2013) revealed an increase of HER2-positive cases, showing that the final change of the new guideline is more related to the change of ISH criteria rather than IHC criteria. Additionally, we also underlined an important fact: only ~50% of the positive cases fulfil both ISH criteria (HER2/CEP17 ratio and average HER2 copy number), highlighting the importance of a control probe in the classification of HER2 gene amplification. Finally, we addressed the issue of equivocal cases (HER2/CEP17 ratio <2.0 and average HER2 copy number ≥4.0 and <6.0 signals *per* cell), which represent a new category not really appreciated by most clinicians. In fact, the equivocal cases should not be compared with the previous "borderline" cases from the 2007 guideline (HER2/CEP17 ratio ≤2.2 and ≥1.8), that just represented cases near the threshold of positivity. In practice, these cases were previously subdivided using the criteria from the first-generation of clinical trials (HER2/CEP17 ratio < or ≥2.0) to decide for targeted-therapy.[155] Currently, an equivocal result by ISH technique requires additional testing to achieve a more definite result using either another block of the same specimen or another specimen (NCB, SES, lymph node or metastatic lesion). If, after this cycle of tests, the result remains to be equivocal, the oncologist may consider HER2-targeted therapy based on patient status and preferences.[97]

The second work aimed to determine the concordance of the HER2 gene amplification assay among different observers by counting increasing numbers of invasive cancer cells. It is remarkable that this question wasn´t done by anyone until now: what is the minimum cell count that allows for acceptable concordance rate and low variability between different measurements or observers? As expected, the increase in cell count from 20 to 60 invasive cells increased the concordance rate between different observers (interobserver) and within the same observer (intraobserver). This performance was mainly due to the decrease of the variability of the

HER2/CEP17 ratio and of the average HER2 copy number measurements. Importantly, we showed that only the observer with more experience could achieve near acceptable agreement rates just by counting the minimum cells recommended by the current ASCO/CAP guideline (20 invasive cells). As such, we propose that the minimum cell number of invasive cells should be raised to at least 40, and preferably 60 invasive cells (in observers with less experience). Additionally, the cases with discordant results presented HER2/CEP17 ratios near the threshold of positivity, making it the most probable cause of discordancy and excluding HER2-GH as the usual culprit. In such cases, the importance of counting more invasive cells is even greater, as well as having a dual count from an experienced observer to guarantee an accurate result. Lastly, we showed that the variability of the HER2/CEP17 ratio was significantly higher in HER2-positive than in HER2-negative cases, given the subjective quantification of the number of HER2 signals in clusters. Nevertheless, it remains open the hypothesis that genetic instability of HER2 gene amplification could exist in separate areas of HER2-positive tumors, as it is expected by a breakage-fusion-bridge (BFB) mechanism (presence of copy number heterogeneity).

The third work aimed to compare the amplification status of the HER2 gene between IC and adjacent CIS, searching for HER2-GH in both components, providing a way to study the relevance of HER2 gene amplification in the progression of CIS to IC. Additionally, we characterized all our cases with HER2-GH in IC since the introduction of the 2013 ASCO/CAP HER2 guideline, also evaluating the amplification status of the HER2 gene in the CIS component if present. This approach could measure the impact of HER2-GH in IC on the result of the amplification test and provide evidence of clonality between IC and CIS. Regarding the first objective, we showed that all IC cases had an adjacent CIS with the same HER2 status, but with 4 cases of CIS presenting HER2-GH. Interestingly, in these 4 cases with HER2-GH in CIS,

we found only 2 IC cases presenting homogeneous HER2 gene amplification. This means that HER2 gene amplification is not relevant for the transition from CIS to IC, given the fact that the remaining 2 IC cases, with adjacent HER2-GH in CIS, had no HER2 gene amplification. It would be expected that the CIS component with HER2 gene amplification, which is significantly correlated with high nuclear grade and aggressive biological behavior, would be the component more likely to become invasive. Nevertheless, we observed that components of CIS without HER2 gene amplification could become invasive even in close proximity with components of CIS with HER2 gene amplification. Additionally, if the trigger for invasion would originate in the stroma we would see both components of CIS, with and without HER2 gene amplification, becoming invasive, which we do not. As such, the trigger for invasion probably originates more often inside the components of CIS itself. Nevertheless, it remains to be explored if HER2-GH in CIS can be clonally related or represent independent lesions colliding with each other. We speculate that probably both situations can occur and at least one of the cases of CIS with HER2-GH represents most likely two independent lesions (low-grade LCIS and high-grade DCIS in case 4 from the 1st cohort). Regarding the second objective, we showed that HER2-GH in the IC cases according to the 2013 ASCO/CAP HER2 guideline is a rare event (about 1% of the cases) introducing low interference on the result of the amplification test in clinical practice. Furthermore, these heterogeneous cases had adjacent CIS without HER2 gene amplification, showing that the development of HER2 gene amplification occurred after invasion. Interestingly, it has been shown that almost always the HER2-positive and HER2-negative components of IC cases with HER2-GH are clonally related, given that their patterns of CNAs are highly similar.[271] Nevertheless, it has also been shown that independent collision tumors can exist creating a single tumor mass with and without HER2 gene amplification.[270]

Finally, the study of stromal TILs and PDL1 expression in BC aimed to measure the importance of these biomarkers in clinical practice. First, we correlate both stromal TILs and PDL1 expression with classical pathological features in addition to molecular subtype and prognosis. We showed that stromal TILs and PDL1 expression in BC were correlated with each other as well as with BC presenting aggressive features. Moreover, in these cases the presence of stromal TILs was significantly associated with better prognosis. Although stromal TILs were not further characterized, it is extraordinary that the quantification of the associated inflammatory response against tumor tissue provides useful information. In future work, we intend to evaluate the clinical impact of the presence of different inflammatory cells in BC tissue, such as B cells, T cells (including cytotoxic and immunosuppressive) and macrophages. Second, we also correlate these biomarkers with CSCs and EMT markers, showing important associations, particularly in PDL1-positive BC cases, providing an important link between stemness and immune resistance. Furthermore, we showed for the first time that the presence of stromal TILs in vimentin-positive BC were independently associated with better prognosis, reflecting an active antitumor immune response. Additionally, in the same setting, PDL1 expression in BC tissue was independently associated with worse prognosis, suggesting that these cases (vimentin+/PDL1+) could be the ones more responsive to immunotherapy. Third, we compared the expression of stromal TILs and PDL1 in both in situ and invasive BC, using pair-matched cases, to undercover their role in the progression from in situ to invasive cancer. We observed a high concordance rate of stromal TILs and PDL1 expression between DCIS and invasive BC, showing expression of PDL1 in DCIS cells for the first time and that the immune response to tumors not only begins at initial stages of carcinogenesis but also that it is probably related with different molecular subtypes,

that possess intrinsic immunogenicity. As such, stromal TILs and PDL1 expression probably are not relevant in the progression from in situ to invasive BC.

CHAPTER 8

CONCLUSIONS AND FUTURE PERSPECTIVES

Regarding the studies performed concerning the oncogene HER2, the conclusions are as follow:

1.1 The application of the 2013 ASCO/CAP guideline increased the number of HER2-positive cases, selecting more patients for anti-HER2 targeted therapy.

1.2 The change of the 2013 ASCO/CAP guideline is mostly due to modifications of ISH criteria rather than of IHC criteria.

1.3 The HER2-positive cases are the primary result of HER2/CEP17 ratio revealing the importance of a control probe in the classification of HER2 gene amplification.

2.1 The increase in cell count improved the intraobserver and interobserver concordance rate of the HER2 test.

2.2 Counting 20 invasive cells is not sufficient and the minimum cell number should be raised to at least 40, and preferably 60 invasive cells.

2.3 The cases with discordant results are more frequent when HER2/CEP17 ratio is near the threshold of positivity.

3.1 All BC cases showed IC and adjacent CIS with the same HER2 gene amplification status.

3.2 According to the latest definition, HER2-GH is a rare event and can be present in both in situ and invasive BC.

3.3 HER2 gene amplification is not relevant in the progression from in situ to invasive BC.

Regarding the study concerning the presence of stromal TILs and PDL1 expression in BC, the conclusions are as follow:

4.1 Stromal TILs and PDL1 expression are associated with high grade and ER-negative BC, as well as with the expression of basal cells markers.

4.2 PDL1 expression in BC is strongly associated with CSCs markers.

4.3 Stromal TILs and PDL1 expression are not relevant in the progression from in situ to invasive BC.

Following the above-mentioned conclusions, we propose novel studies to highlight the role of these tumor-based biomarkers in prognostication of BC:

1. Study the concordance of HER2 gene amplification test between different ISH techniques (FISH and SISH) with the application of the new guideline to clarify its role in the classification of equivocal results.

2. Study the quantification of HER2 gene amplification by imaging analysis (in ISH) and by molecular techniques to determine HER2 gene amplification more accurately.

3. Study the clonal relationship of HER2-positive and HER2-negative components of HER2-GH in both IC and CIS.

4. Study the clinical impact of B cells, T cells, including cytotoxic (CD8+) and immunosuppressive (CD4+/FOXP3+), and macrophages (M1 and M2) in BC tissue.

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Papers

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ORIGINAL ARTICLE

Application of the 2013 ASCO/CAP guideline and the SISH technique for HER2 testing of breast cancer selects more patients for anti-HER2 treatment

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Abstract The aim of this study is to assess the impact of changes of the 2013 ASCO/CAP guideline on the results of HER2 testing in breast cancer. A series of 916 primary invasive breast cancer cases, assessed as HER2 2+ by IHC in part using the 2007 and in part the 2013 ASCO/CAP criteria, was evaluated for HER2 amplification status by SISH and classified according to both 2007 and 2013 ASCO/CAP ISH guideline criteria. We observed a significant increase of HER2-positive cases (12.4 to 16.8 %) and a decrease of HER2-equivocal cases (3.6 to 0.7 %). Of the cases studied, 52.1 % fulfilled both criteria of HER2/CEP17 ratio and average HER2 copy number per cell to be classified as HER2-positive. Reclassification of the cases from before the introduction of the new ASCO/CAP guideline with the 2013 ISH criteria resulted in an increase of cases with a HER2-positive status (12.4 to 14.2 %) and in a decrease of HER2-equivocal cases (3.6 to 1.6 %). The 2013 ASCO/CAP guideline selects more patients for anti-HER2 targeted therapy, mostly based on the modifications of criteria to evaluate ISH-HER2.

Keywords ASCO/CAP · Breast cancer · HER2 · SISH

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Introduction

In the western world, breast cancer (BC) is the most commonly diagnosed malignancy among women, representing about 30 % of all new cancer cases, and after lung cancer the second leading cause of cancer death [1, 2]. The current cancer care guidelines for BC recommend that estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor 2 (HER2) status must be routinely determined in all patients with invasive BC, BC recurrence and BC metastases [3, 4]. These guidelines were published to help improve laboratory performance in the determination of these markers, which provide useful predictive information regarding response to targeted therapy.

HER2, located on the long arm of chromosome 17 (17q12), is amplified and/or overexpressed in about 15 to 20 % of invasive BC. Cases with a HER2-positive status represent a clinically important subset of BC associated with poor outcome but also with a high likelihood of response to HER2-targeted therapy [5–8]. Several studies have shown that anti-HER2 therapy given during and/or after chemotherapy results in a significant improvement in disease-free and overall survival [9–11]. Therefore, HER2 is a helpful marker for therapy decision making in patients with BC and appropriate evaluation of HER2 status ensures that the right patient receives the right treatment [3].

At present, HER2 protein expression is determined in BC samples by immunohistochemistry (IHC) resulting in three possible outcomes: negative (score 0 or 1+), equivocal (score 2+), and positive (score 3+). If the IHC result is equivocal, reflex testing should be performed on the same specimen using an alternative assay, such as in situ hybridization (ISH) [3].

The new 2013 ASCO/CAP (American Society of Clinical Oncology/College of American Pathologists) guideline has

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updated the definition of HER2-positive status by modifying both IHC and ISH criteria, reducing the thresholds for post-analytical interpretation of positive results in comparison with the previous 2007 ASCO/CAP guideline [3, 12]. In the new guideline, a HER2 score 3+ is defined as the presence of complete and intense membrane staining, in at least 10 % of tumor cells [3]. This represented a return to the IHC criteria originally used in the first-generation clinical trials [13]. A similar approach was used regarding ISH criteria (see below).

In this study, we aim to compare the impact of the change from the 2007 to the 2013 ASCO/CAP guidelines on the result of HER2 amplification test in BC.

Materials and methods

Cases

A series of 916 primary invasive BC cases was retrieved from the archives of Ipatimup Diagnostics, including cases evaluated 1 year before (494 cases from November 2012 to October 2013) and 1 year after (422 cases from December 2013 to November 2014) the publication of the new ASCO/CAP guideline (November 2013). All BC cases (core biopsies and surgical specimens) had been fixed in 10 % formalin, embedded in paraffin, and were referred to our institution (national reference center for HER2 ISH) with an equivocal IHC HER2 score (2+) to perform the HER2 amplification assay with a HER2 Dual ISH DNA Probe with a silver marker (SISH).

Ethics approval and informed consent were not required for this study.

SISH

SISH testing was performed on 3-μm sections of formalin-fixed, paraffin-embedded tissue of all BC cases using dual-hapten, dual-color ISH (DDISH). The dual-probe assay (INFORM HER2 Dual ISH DNA Probe Cocktail Assay; Ventana Medical Systems, Inc., Tucson, Arizona) contains a HER2 locus-specific probe and a control probe specific for the centromere of chromosome 17 (CEP17). The entire procedure was carried out on an automated staining system (Ventana BenchMark™ XT Staining System) according to the manufacturer's instructions. Positive and negative controls were used for each staining run.

Evaluation of the results included recording the number of HER2 and CEP17 signals in at least 20 nuclei in two different areas. The samples were classified by pathologists (AP and FS) according to the 2007 and 2013 ISH criteria for HER2 amplification. Corresponding hematoxylin and eosin staining were used for the identification of the invasive component of the tumor.

The 2007 ASCO/CAP guideline defines HER2 amplification as positive at a HER2/CEP17 ratio >2.2, equivocal at a HER2/CEP17 ratio ≤2.2 and ≥1.8, and negative at a HER2/CEP17 ratio <1.8 [12]. The 2013 ASCO/CAP guideline establishes the result of HER2 amplification as positive at a HER2/CEP17 ratio ≥2.0 or a HER2/CEP17 ratio <2.0 and an average HER2 copy number per cell of ≥6.0, equivocal when HER2/CEP17 ratio <2.0 and average of HER2 copy number ≥4.0 and <6.0 signals per cell, and negative when HER2/CEP17 ratio <2.0 and average HER2 copy number of <4.0 signals per cell [3].

Chromosome 17 polysomy was defined as an average of ≥3.0 CEP17 signals per cell [14]. Genomic heterogeneity was also recorded and considered present if a discrete population of tumor cells with HER2 amplification represented at least 10 % of the total tumor cell population [3].

Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 21.0 for Windows. The Pearson's chi-square (χ^2) test and McNemar test were used for comparison of qualitative variables and the *t* test for quantitative variables. The level of significance was set at $p < 0.05$.

Results

The 916 BC cases concerned 97.2 % women and 1.2 % men. The age ranged from 24 to 103 years, with a median age at diagnosis of 59 years.

The distribution of gender, age, HER2/CEP17 ratio, and average HER2 copy number per cell were not statistically different between the pre- and post-new guideline cases (Table 1 and Fig. 1). The only parameters that changed significantly with the new guideline were the average CEP17 copy number per cell and the presence of chromosome 17 polysomy (4.1 to 0.9 %; $p = 0.003$; Table 1).

Table 2 and Fig. 2a present the results of HER2 test performed on the pre-new guideline cases (using the ISH criteria from the 2007 ASCO/CAP guideline): 415 cases (84.0 %) HER2-negative, 18 cases (3.6 %) HER2-equivocal, and 61 cases (12.4 %) HER2-positive. Table 2 and Fig. 2b present the results of HER2 test performed on the post-new guideline cases (using the ISH criteria from the 2013 ASCO/CAP guideline): 348 cases (82.5 %) HER2-negative, 3 cases (0.7 %) HER2-equivocal, and 71 cases (16.8 %) HER2-positive. The differences are statistically significant (Table 2—statistical analysis A; $p = 0.003$). We also observed that 52.1 % of the positive cases (37/71) fulfill both criteria of HER2/CEP17 ratio ≥2.0 and average of HER2 copy number per cell ≥6.0 (Table 3 and Fig. 2b). We furthermore classified the pre- and

Table 1 Differences between the cases before and after the introduction of the 2013 ASCO/CAP guideline

	Cases before 2013ASCO/ CAP guideline	Cases after 2013ASCO/ CAP guideline	*p*
Gender (female/male/NI)	481/6/7	409/5/8	ns (0.974)[a]
Age (mean ± sd)	58.17 ± 13.76	59.12 ± 14.10	ns (0.346)[b]
HER2/CEP17 ratio (mean ± sd)	1.68 ± 1.57	1.68 ± 1.50	ns (0.930)[b]
Average of HER2 copy number per cell (mean ± sd)	3.17 ± 2.56	2.88 ± 2.42	ns (0.077)[b]
Average of CEP17 copy number per cell (mean ± sd)	1.98 ± 0.51	1.78 ± 0.42	<0.001[b]
Chromosome 17 polysomy (present/absent)	20 (4.1 %)/474 (95.9 %)	4 (0.9 %)/418 (99.1 %)	0.003[a]

NI not informed, *ns* not significant

[a] Pearson chi-square test

[b] *t* test

post-new guideline cases using the 2007 and 2013 ISH criteria and observed a slight but non-significant increase in HER2-positive cases and a similar decrease in HER2-equivocal cases (Table 2—statistical analysis B and C; $p = 0.185$ and $p = 0.261$, respectively).

In the reclassification of the two case series using the 2007 and 2013 ISH criteria, we observed an increase in HER2-positive cases (12.4 to 14.2 % and 15.9 to 16.8 %, respectively) and a decrease in HER2-equivocal cases (3.6 to 1.6 % and 2.4 to 0.7 %, respectively). This was statistically significant in the pre-new guideline cases (Table 2—statistical analysis D; $p = 0.011$) and near significant in post-new guideline cases (Table 2—statistical analysis E; $p = 0.071$).

In the pre-new guideline cases, the 2013 ISH criteria reclassified 22 (4.5 %) of the cases, 9 as HER2-positive (from HER2-equivocal), 7 as HER2-negative (from HER2-equivocal), and 6 as HER2-equivocal (from HER2-negative). All HER2-positive cases according to the 2007 guideline remained HER2 positive with the 2013 guideline (Table 4).

In the post-new guideline cases, genomic heterogeneity was detected in 0.47 % of the cases (2/422), the proportion of HER2 amplified cells varying between 25 and 40 % of the tumor cell population.

Discussion

Our center (Ipatimup) is one of the reference centers for SISH test of BC in Portugal. In our center, the introduction of the updated ASCO/CAP guideline for HER2 test by SISH resulted in a significant increase of positive cases (12.4 to 16.8 %) and decrease of equivocal cases (3.6 to 0.7 %).

Several studies recently reported an increase of HER2-positive cases evaluated by FISH but also an increase of

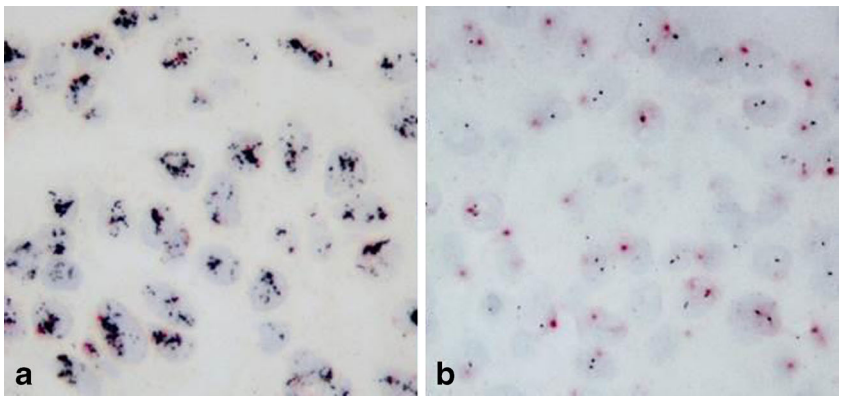

Fig. 1 Examples of results of HER2 detection by SISH technique (×400). **a** HER2-positive; **b** HER2-negative

Table 2 Classification of HER2 test according to the 2007 and 2013 ISH criteria

HER2 result	Cases before 2013ASCO/CAP guideline		Cases after 2013ASCO/CAP guideline	
	ISH criteria 2007	ISH criteria 2013	ISH criteria 2007	ISH criteria 2013
Positive	12.4 % (61)	14.2 % (70)	15.9 % (67)	16.8 % (71)
Equivocal	3.6 % (18)	1.6 % (8)	2.4 % (10)	0.7 % (3)
Negative	84.0 % (415)	84.2 % (416)	81.7 % (345)	82.5 % (348)
Total	494		422	
Statistical analysis	A			A
	B		B	
		C		C
	D	D	E	E

A cases before (ISH criteria 2007) vs after (ISH criteria 2013) 2013 ASCO/CAP guideline: $p = 0.003$[a]; *B* cases before vs after 2013ASCO/CAP guideline (ISH criteria 2007): $p = 0.185$[a]; *C* cases before vs after 2013ASCO/CAP guideline (ISH criteria 2013): $p = 0.261$[a]; *D* cases before 2013ASCO/CAP guideline—ISH criteria 2007 vs ISH criteria 2013: $p = 0.011$[b]; *E* cases after 2013ASCO/CAP guideline—ISH criteria 2007 vs ISH criteria 2013: $p = 0.071$[b]

[a] Pearson Chi-Square test

[b] McNemar test

HER2-equivocal cases with the introduction of 2013 ASCO/CAP guideline [15–19]. However, the study by the group of Garbar et al. had results similar to ours using FISH, with an increase of HER2-positive cases and a slight decrease in HER2-equivocal cases [20]. The explanation for these differences is not clear, but this might be related to the number of cases, pre-analytical conditions, and different ISH platforms. We did not review centrally the IHC performed externally, which might explain the decrease in equivocal cases in comparison with recent literature.

As yet, the published concordance rates between SISH and FISH vary between 92 and 99 %, the majority fulfilling the ASCO/CAP validation requirement of a concordance rate exceeding 95 % (Table 5) [21–30]. However, the requirement in the 2013 ASCO/CAP guideline to determine the average of HER2 copy number (first applied to bright field ISH and now applied to the FISH test) introduces a problem that did not exist previously. Autofluorescence in FISH might result in overestimation of both HER2 and CEP17 signals, resulting in HER2/CEP17 ratios below 2.0 and average of HER2 copy numbers above 4 per cell and an increase of equivocal HER2 results [31, 32]. If an increase of HER2-equivocal cases by FISH and a decrease of HER2-equivocal cases by SISH is confirmed, the concordance rate of these two ISH tests might decrease to under 95 %. This would open up the question which of these techniques provides the most reliable information on HER2 amplification status.

For nearly half of the cases studied (52.1 %), both criteria (HER2/CEP17 ratio and average of HER2 copy number per cell) were fulfilled to allow them to be classified as HER2-

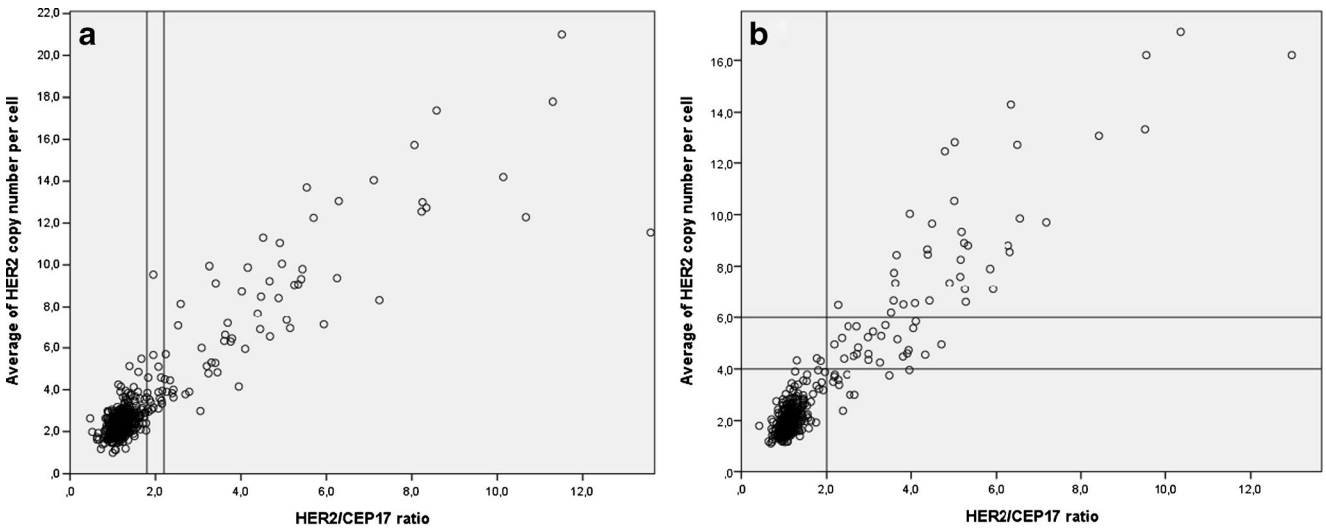

Fig. 2 Cases before (**a**) and after (**b**) the introduction of the 2013 ASCO/CAP guideline

Table 3 Classification of the cases after the 2013 ASCO/CAP guideline

HER2/CEP17 ratio	Average of HER2 copy number signals per cell		
	<4.0	≥4.0 and <6.0	≥6.0
<2.0	348	3	0
≥2.0	11	23	37

positive. This is particularly relevant given the fact that half the cases would be excluded from targeted therapy if HER2 amplification would be evaluated using just the HER2 probe (as some methods do).

Classification of the pre-new guideline and post-new guideline case series with the 2007 and 2013 criteria did not result in significant changes in the HER2 test results. This suggests that modifying the threshold in IHC, from 30 to 10 % of cells with moderate staining, had little effect on the HER2 amplification test results. Lee et al. found that cases with equivocal IHC (score 2+) in 10–30 % of the cells had a probability of being amplified of 5–12 % [33]. It is then not surprising that inclusion of these cases does not significantly change the HER2 amplification test results.

In contrast, classification of pre-new guideline and post-new guideline cases with different ISH criteria (2007 and 2013) resulted in significant changes in HER2 amplification test results. Our findings suggest that the 2013 modified ISH criteria had a stronger impact on the test results than the modified IHC criteria. We found that the 2013 ISH criteria resulted in reclassification of 4.5 % of the cases. Other publications have shown a reclassification rate of up to 15 % of cases [16, 17].

Polysomy of chromosome 17 changed from 4.1 to 0.9 % with the introduction of the 2013 ASCO/CAP guideline, which is probably due to modification of the definition of equivocal IHC HER2 staining (score 2+) rather than a change in the biology of the tumors. Several studies have shown that polysomy of chromosome 17 (measured on the basis of CEP17) varies between 3 and 49 % of the cases, depending on the definitions of polysomy and on the method used [12, 14, 34, 35]. The approach is based on the notion that CEP17 copy number is a surrogate marker for chromosome 17 copy number. However, molecular karyotyping has revealed that an increased CEP17 signal number is usually due to gain of the pericentromeric region rather than to duplication of the entire chromosome [36–41]. CEP17 might therefore not be a good marker for polysomy 17, making true polysomy 17 probably a rare event in BC. Nevertheless, CEP17 amplification can still be the cause of misleading HER2 amplification and false-negative test results, excluding patients from anti-HER2 targeted therapy [34].

Table 4 Classification of the cases before the 2013 ASCO/CAP guideline

2007 ISH criteria	2013 ISH criteria			
	Positive	Negative	Equivocal	Total
Positive	61	0	0	61
Negative	0	409	6	415
Equivocal	9	7	2	18
Total	70	416	8	494

Table 5 Concordance rates of SISH vs FISH according to the 2007 ASCO/CAP guideline

Publication	Concordance (%)	Year
Dietel et al. [21]	96	2007
Shousha et al. [22]	94	2009
Bartlett et al. [23]	96	2009
Papouchado et al. [24]	98.9	2010
Koh et al. [25]	97	2011
Lee et al. [26]	96.7	2011
Park et al. [27]	96.5	2012
Jacquemier et al. [28]	97	2013
Lim et al. [29]	93	2013
Unal et al. [30]	92.3	2013

Tumors with polysomy 17 are thought to be different from non-HER2 amplified tumors, associated with a more aggressive clinical behavior and not responsive to conventional therapy [14, 42]. However, in BC, the relationship between polysomy of chromosome 17 and the response to anti-HER2 therapy remains to be determined [43–45].

We found the presence of genomic heterogeneity to be rare as observed in just 0.47 % of cases. Several studies have addressed this issue in the past and reported genomic heterogeneity in 5 to 40 % of BC cases [14, 46–49]. Studies on the relationship between genomic heterogeneity and prognosis have shown that tumors with a HER2 amplification in at least 30 % of the cells have a reduced disease-free survival [48, 49]. However, the definition of genomic heterogeneity has also changed from individual cells (between 5 and 50 % of tumor cells with HER2 amplification) to discrete populations of tumor cells (at least 10 % of the total tumor cell population with HER2 amplification) [3, 50]. Additional work is needed to determine the prevalence of genomic heterogeneity with this new definition and the response to anti-HER2 targeted therapy in these patients.

In conclusion, we show that the new HER2 guideline results in an increased number of HER2-positive and a decreased number of HER2-equivocal cases using the SISH technique, primarily because of modifications of ISH rather than of IHC criteria. As a consequence, the 2013 ASCO/CAP guideline selects more patients for anti-HER2 targeted therapy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing of interests.

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Counting invasive breast cancer cells in the *HER2* silver *in-situ* hybridization test: how many cells are enough?

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Counting invasive breast cancer cells in the *HER2* silver *in-situ* hybridization test: how many cells are enough?

Aim: To evaluate the intraobserver and interobserver reproducibility of the *HER2 in-situ* hybridization (ISH) test in breast cancer by measuring the impact of counting different numbers of invasive cancer cells.

Methods and results: A cohort of 101 primary invasive breast cancer cases were evaluated for *HER2* gene amplification by silver ISH, and the concordance among four observers with different levels of experience, counting different numbers of invasive cancer cells, was determined. The evaluation of the samples included scoring 20 nuclei, in three different areas. The cases were scored twice, with a washout interval of at least 2 weeks. We observed an increase in the intraobserver concordance rate between the first and second evaluations with an increase in cell count. A count of 60 invasive cells was needed to obtain a concordance rate near 95% and an agreement rate greater than 0.80 by all observers. The interobserver concordance rate of the *HER2* test also increased with the increase in cell count, reaching at least a 90% concordance rate with a count of 60 invasive cells. The median variability of both the *HER2*/CEP17 ratio and the average *HER2* copy number between different evaluations decreased with the increase in cell count, being statistically higher in *HER2*-positive cases.

Conclusions: The minimal cell number recommended in current guidelines should be raised to at least 40, and preferably 60, invasive cells. Moreover, cases with amplification levels close to the threshold should be subjected to a dual count from an experienced observer.

Keywords: ASCO/CAP, breast cancer, HER2, SISH

Introduction

HER2 is a transmembrane protein receptor with tyrosine kinase activity, and is amplified and/or overexpressed in approximately 15–20% of invasive breast cancers (BCs).[1–3] Numerous clinical trials have demonstrated that HER2-targeted therapy improves progression-free survival and overall survival only in patients with BCs showing *HER2* amplification.[4–7] For this reason, the accurate assessment of *HER2* amplification identifies patients who are most likely to benefit from targeted therapy.

HER2 evaluation is most frequently performed by immunohistochemistry (IHC), resulting in three possible outcomes: negative (score of 0 or 1+), equivocal (score of 2+), and positive (score of 3+). In cases of

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equivocal results, reflex testing should be performed with *in-situ* hybridization (ISH) assays for the assessment of *HER2* amplification.[8]

Regarding ISH assays, the 2013 American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines accept bright-field ISH and recommend counting at least 20 non-overlapping cells in two separate areas of invasive cancer.[8] Although this is usually interpreted as counting a total of 40 cells (at least 20 cells per area), the supplementary data of the guidelines actually explain that the minimum cell number is, in fact, a total of 20 cells in two separate areas of invasive cancer (at least 10 cells per area). It has already been shown that high ISH interobserver reproducibility exists; nevertheless, the minimum number of cells that should be counted to obtain a reproducible result is yet to be determined.[9,10] Even if the current ASCO/CAP guideline recommendations are adhered to, the imprecision of HER2 testing remains a relevant issue, for both IHC and ISH techniques.[11,12]

In the present study, *HER2* amplification status was determined in a series of primary BC cases by four different observers, who scored the cases twice according to the 2013 ASCO/CAP guidelines for HER2 testing. Specifically, we aimed to evaluate the intraobserver and interobserver interpretative reproducibility of the *HER2* assay in BCs by using bright-field ISH to evaluate the impact of counting different numbers of invasive cancer cells.

Materials and methods

CASES

A cohort of 101 consecutive primary invasive BC cases was retrieved from the archives of Ipatimup Diagnostics from April to June 2015 to determine the concordance of the *HER2* amplification assay among four observers counting different numbers of invasive cancer cells.

The cases included formalin-fixed paraffin-embedded core biopsies and surgical specimens referred to our institution with an equivocal HER2 result by IHC (score of 2+) for performance of an evaluation of *HER2* amplification with bright-field ISH. All cases were reviewed for diagnosis and histological grade. Ethical approval and informed consent were not required for this study.

SILVER ISH

Silver ISH was performed on 3-μm-thick sections with dual-hapten, dual-colour ISH. The dual-probe assay (INFORM HER2 Dual ISH DNA Probe Cocktail Assay; Ventana Medical Systems, Tucson, AZ, USA), which is Food and Drug Administration-approved, contains an *HER2* locus-specific probe (black signal) and a control probe specific for the centromere of chromosome 17 [centromere enumeration probe 17 (CEP17), red signal] that allows detection of *HER2* amplification by light microscopy. The entire procedure was carried out on an automated staining system (Ventana BenchMark XT Staining System; Ventana Medical Systems, Tucson, AZ, USA) according to the manufacturer's instructions. Appropriate positive and negative controls were used in every set of slides. Optimal staining consists of an absence of non-specific background staining, distinct nuclear morphology, and clear and specific signals within the nucleus.

SISH INTERPRETATION

The samples were classified by two pathologists (A.P. and C.E.) and two pathology residents (J.P. and A.B.) according to the 2013 ASCO/CAP ISH criteria for *HER2* amplification. Pathologists had different levels of experience, one with >500 cases evaluated per year (A.P.) and the other with <50 cases evaluated per year (C.E.). Both pathology residents had attended a previous molecular pathology training course, where they learned how to perform SISH interpretation. Corresponding haematoxylin and eosin staining was used for the identification of the invasive component of the tumour, and only cells with a minimum of one copy of *HER2* and CEP17 each were scored. The number of *HER2* signals was estimated in clusters, except for doublets, which counted as a single signal.

The evaluation of the samples included scoring 20 nuclei, in three different areas, and recording the numbers of *HER2* and CEP17 signals in groups of 10 invasive cancer cells. This approach allows us to add groups of 10 cells in each area to groups of 10 cells in other areas, giving 12 results of scoring 20 cells in two separate areas (10 cells per area) (Figure 1). The same can be applied to the evaluation of scoring 40 cells in two separate areas (20 cells per area, adding the first area to the second and third areas, and the second area to the third area), creating three results. Finally, the three areas can be added, generating one result of scoring 60 cells per case. The cases were scored twice with a washout interval of at least 2 weeks, in a blinded mode.

The 2013 ASCO/CAP guidelines establish the result of *HER2* amplification as: positive when the *HER2*/CEP17 ratio is ≥2.0 or <2.0, and the average *HER2*

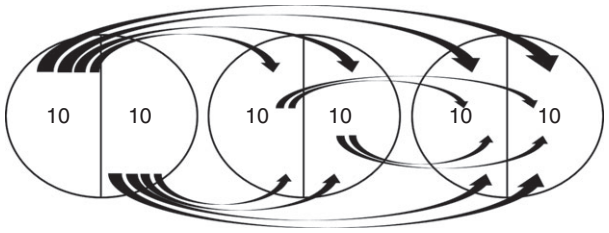

Figure 1. Scoring 20 cells in two separate areas (10 cells per area), giving 12 results.

Table 1. Cohort characteristics

Characteristic	Value
Procedure (core biopsy/surgical specimen) (*n*)	82/19
Gender (female/male) (*n*)	98/3
Age (years) (mean ± SD)	63.67 ± 15.77
Histological type (*n*)	
Invasive carcinoma, NST	94
Lobular carcinoma	5
Mucinous carcinoma	2
Histological grade (*n*)	
Grade 1/Grade 2/Grade 3	12/62/27

NST, No special type; SD, Standard deviation.

copy number is ≥6.0 signals per cell; equivocal when the *HER2*/CEP17 ratio is <2.0, and the average *HER2* copy number is ≥4.0 and <6.0 signals per cell; and negative when the *HER2*/CEP17 ratio is <2.0, and the average *HER2* copy number is <4.0 signals per cell.[8]

Cases with discordant results were reviewed by both pathologists during a common microscopy session to document genomic heterogeneity, defined in the latest ASCO/CAP guidelines as a discrete population of tumour cells with *HER2* amplification.[8] No additional testing was performed to resolve the discordance.

Chromosome 17 polysomy was defined as an average of ≥3.0 CEP17 signals per cell.[13]

STATISTICAL ANALYSIS

Statistical analyses were performed with SPSS version 24.0 for Windows. Pearson's χ^2 test (or Fisher's exact test, if appropriate) was used for comparison of qualitative variables, and the Mann–Whitney *U*-test (MWUT) and Pearson's correlation coefficient (PCC) were used for comparison of quantitative variables. The level of significance was set at $P < 0.05$.

Agreement rates of the same observer (intraobserver) and those between each observer (interobserver) regarding interpretation of the *HER2* amplification assay were evaluated with kappa (*k*) statistics. *k*-Values range between zero (chance agreement) and 1 (perfect agreement), and were considered to be satisfactory if they were >0.80.

Results

The cohort included 82 core biopsies and 19 surgical specimens, with 97.03% of the cases being diagnosed in women and 2.97% in men. The age of the patients ranged from 35 to 93 years, with a median age at diagnosis of 65 years. The majority of the histological types were invasive carcinomas of no special type, with 11.88% of the cases being classified as grade 1, 61.39% as grade 2, and 26.73% as grade 3. The cohort characteristics are summarized in Table 1.

The final classification of *HER2* testing from each observer (first and second evaluations of 60 invasive cancer cells) varied between 23% and 26% of *HER2*-positive cases (Table 2); there were no cases with genomic heterogeneity. Moreover, no equivocal results or chromosome 17 polysomy were reported by any observer. The intraobserver concordance rate of

Table 2. Classification of HER2 test (first and second evaluation of 60 cells per case)

	Pathologist 1		Pathologist 2		Resident 1		Resident 2	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Positive, *n* (%)	25 (24.8)	25 (24.8)	23 (22.8)	23 (22.8)	25 (24.8)	24 (23.8)	25 (24.8)	26 (25.7)
Negative, *n* (%)	76 (75.2)	76 (75.2)	78 (77.2)	78 (77.2)	76 (75.2)	77 (76.2)	76 (75.2)	75 (74.3)
Equivocal (*n*)	0	0	0	0	0	0	0	0
Total (*n*)	101							

Table 3. Intraobserver concordance rate of *HER2* test results with different cell counts

	Pathologist 1			Pathologist 2			Resident 1			Resident 2		
Cells	1st	2nd	1st versus 2nd	1st	2nd	1st versus 2nd	1st	2nd	1st versus 2nd	1st	2nd	1st versus 2nd
20	96.04–100% (0.891–1.000)	95.05–100% (0.862–1.000)	93.07–100% (0.806–1.000)	93.07–100% (0.800–1.000)	91.09–100% (0.758–1.000)	86.14–97.03% (0.632–0.911)	95.05–100% (0.868–1.000)	96.04–100% (0.891–1.000)	92.08–99.01% (0.786–0.973)	86.14–99.01% (0.638–0.972)	86.14–100% (0.662–1.000)	76.24–96.04% (0.442–0.896)
40	99.01–100% (0.973–1.000)	98.02–100% (0.947–1.000)	97.03–100% (0.919–1.000)	96.04–98.02% (0.887–0.944)	98.02–99.01% (0.945–0.972)	90.10–95.05% (0.727–0.861)	99.01–100% (0.973–1.000)	100% (1.000)	98.02–99.01% (0.945–0.973)	94.06–97.03% (0.842–0.926)	98.02–100% (0.949–1.000)	90.10–95.05% (0.752–0.869)
60	NA	NA	100% (1.000)	NA	NA	94.06% (0.831)	NA	NA	99.01% (0.973)	NA	NA	95.05% (0.869)

NA, not applicable.
Minimal and maximal concordance rate (*k* statistics).

the *HER2* test with different cell counts is shown in Table 3. We found, for all observers, an increase in the concordance rate between the first and second evaluations with the increase in cell count from 20 to 60 invasive cells [93.07–100%, 86.14–94.06%, 92.08–99.01% and 76.24–95.05% for pathologist 1 (P1), pathologist 2 (P2), resident 1 (R1) and resident 2 (R2), respectively]. P1 was the only observer who reached an intraobserver agreement rate between the first and second evaluations of >0.80 (0.806) just by counting 20 invasive cells; the same goal was achieved by R1 when counting 40 invasive cells (0.945). Additionally, both P1 and R1 needed to count at least 40 invasive cells to achieve a concordance rate between the first and second evaluations of >95% (97.03% and 98.02%, respectively). On the other hand, P2 and R2 needed to count 60 invasive cells to reach an intraobserver agreement rate of >0.80 (0.831 and 0.869, respectively) and a concordance rate of ~95% (94.06% and 95.05%, respectively). We also found that the concordance rates for different areas within the first or second evaluations were always higher than those measured between different evaluations. The same trend was observed in the intraobserver correlation of both the *HER2*/CEP17 ratio and the average *HER2* copy number, whereby it increased with the increase in cell count and was always higher for different areas within each evaluation than for different evaluations (Tables S1 and S2).

The minimal interobserver concordance rate of the *HER2* test increased with the increase in cell count from 20 to 60 invasive cells (73.27% in the P2–R2 evaluation of 20 cells, to 97.03% in the P1–R1 evaluation of 60 cells) (Table 4). A concordance rate of 95.05% and an agreement rate of 0.861 were achieved by P1 and R1 with a count of 40 invasive cells. A count of 60 invasive cells produced a minimal interobserver concordance rate of 90.10% and an agreement rate of 0.734 (R1–R2). In parallel, the same trend described above was found for the correlation of both *HER2*/CEP17 ratio and average *HER2* copy number, in which it increased with the increase in cell count (Tables S3 and S4).

The discordant cases of the observers with higher interobserver concordance or agreement rates (P1 and R1) showed an *HER2*/CEP17 ratio of between 1.39 and 2.72 when 20 invasive cells were counted, of between 1.75 and 2.37 when 40 invasive cells were counted, and of between 1.82 and 2.24 when 60 invasive cells were counted (Table 5; Figure 2). The range of average *HER2* copy number in discordant cases for each observer with different cell counts

Table 4. Interobserver concordance rate of *HER2* test results with different cell counts

	Pathologist 2	Resident 1	Resident 2	Cells
Pathologist 1	87.13–98.02% (0.628–0.945)	92.08–99.01% (0.776–0.974)	77.23–97.03% (0.461–0.921)	20
	91.09–95.05% (0.734–0.865)	95.05–99.01% (0.861–0.973)	88.12–97.03% (0.690–0.921)	40
	93.07–95.05% (0.806–0.865)	97.03–98.02% (0.919–0.947)	92.08–97.03% (0.787–0.921)	60
Pathologist 2		81.19–98.02% (0.515–0.946)	73.27–96.04% (0.386–0.884)	20
		89.11–97.03% (0.704–0.919)	89.11–95.05% (0.695–0.861)	40
		91.09–96.04% (0.750–0.891)	91.09–94.06% (0.751–0.841)	60
Resident 1			77.23–95.05% (0.461–0.869)	20
			88.12–96.04% (0.681–0.894)	40
			90.10–95.05% (0.734–0.869)	60

Minimal and maximal concordance rate (*k* statistics).

is shown in Table S5. Additionally, we observed at least one discordant case for any observer in 39.02% of core biopsies (32/82) and in 10.53% of surgical specimens (2/19) when 20 invasive cells were counted (χ^2 test; $P = 0.018$), and in 14.63% of core biopsies (12/82) and in 10.53% of surgical specimens (2/19) when 60 invasive cells were counted (Fisher's exact test; $P = 1.000$).

The median variability of the *HER2*/CEP17 ratio between different evaluations decreased with the increase in cell count from 20 to 60 invasive cells in both negative cases (0.36–0.06, 0.47–0.15, 0.38–0.08 and 0.54–0.19 for P1, P2, R1 and R2, respectively) and positive cases (1.83–0.50, 1.04–0.31, 1.32–0.27 and 2.44–0.66 for P1, P2, R1 and R2, respectively) (MWUT; $P < 0.001$ for all observers). Furthermore, the median variability of the *HER2*/CEP17 ratio in *HER2*-positive cases was statistically higher than that in *HER2*-negative cases (MWUT; $P < 0.001$ for all observers) (Table 6). In discordant cases the median variability of the *HER2*/CEP17 ratio presented values between those of HER2-negative and HER2-positive cases and decreased with the increase in cell count (0.65–0.40, 1.01–0.93, 0.96–0.42 and 1.13–0.63 for P1, P2, R1 and R2, respectively), with the majority being statistically different from negative cases, positive cases, or both (Table 7). Figure 3 shows the relationship of the average of the *HER2*/CEP17 ratio for all observers between the first and second evaluations with its variability when 60 invasive cells were counted, confirming the increased variability in cases with a higher *HER2*/CEP17 ratio (PCC 0.881; $P < 0.001$). We also found that, for all observers, the median variability of the *HER2*/CEP17 ratio between different areas within the first and second evaluations was similar (MWUT; $P > 0.05$) and always lower than the variability between different evaluations (MWUT; $P < 0.05$). The same pattern described above was observed in the variability of the average *HER2* copy number with different cell counts in negative, positive and discordant cases (Tables S6 and S7).

Finally, when we compared the variability of the *HER2*/CEP17 ratio with the variability of the average *HER2* copy number, we observed that the former was always inferior to the latter (MWUT; $P < 0.05$ for all observers when counting 60 invasive cells) (Tables 6 and S6).

Discussion

Since the introduction of the new ASCO/CAP guidelines for *HER2* testing in BC, numerous studies have reported an increase in the number of *HER2*-positive cases.[14–18] Recently, using SISH, we showed that the updated ASCO/CAP guidelines resulted in a significant increase in the number of *HER2*-positive cases and a decrease in the number of equivocal cases.[19] Most of the published literature shows the concordance rates between SISH and fluorescence ISH to be >90%, and to almost always fulfil the ASCO/CAP validation requirement of a concordance rate of >95%.[20–22]

In the present study, we found an increase in the concordance rate of *HER2* testing between the first and second evaluations with an increase in cell count from 20 to 60 invasive cells, as well as a decrease in

Table 5. Minimum and maximum *HER2*/CEP17 ratio in discordant cases with different cell counts

	Pathologist 1			Pathologist 2			Resident 1			Resident 2		
Cells	1st	2nd	1st versus 2nd	1st	2nd	1st versus 2nd	1st	2nd	1st versus 2nd	1st	2nd	1st versus 2nd
20	1.66–2.41	1.70–2.72	1.66–2.72	1.32–4.37	1.21–4.25	1.21–4.37	1.39–2.68	1.65–2.56	1.39–2.68	1.22–5.13	0.79–3.00	0.79–5.13
40	1.98–2.05	1.89–2.25	1.89–2.25	1.57–2.13	1.71–2.24	0.99–3.44	1.94–2.15	NA	1.75–2.37	1.56–2.42	1.80–2.00	1.56–3.32
60	NA	NA	NA	NA	NA	1.02–3.34	NA	NA	1.82–2.24	NA	NA	1.56–2.94

NA, not applicable.

the variability of *HER2*/CEP17 ratios for all observers, which demonstrated the value of counting additional cells. The fact that only the observer with more experience (P1) could achieve an intraobserver agreement rate of >0.80 between both evaluations when counting only 20 invasive cells shows that 20 cells might not be the optimal minimal cell number as recommended by current guidelines. In fact, P1 and R1 needed to count at least 40 invasive cells to reach an intraobserver concordance rate of >95%, and P2 and R2 needed to count 60 invasive cells to achieve the same rate. Additionally, no observer reached an interobserver concordance rate of >95% or an agreement rate of at least 0.80 by counting only 20 invasive cells. In fact, only two observers (P1 and R1) achieved that aim, and required a minimal count of 40 invasive cells. The remaining observers required a count of 60 invasive cells to achieve concordance rates of >90% of the cases and agreement rates near 0.80. According to our data, the minimal cell number of 20 invasive cells in *HER2* testing in BC is not sufficient, and should be raised to at least 40 invasive cells (which is probably already the case in most laboratories) and, preferably, 60 cells if available, which is almost always manageable in most of the cases in our experience.

Interestingly, the observers with higher interobserver concordance/agreement rates were those with better intraobserver concordance/agreement rates. Additionally, the major causes of discordance in these observers were tumours with an *HER2*/CEP17 ratio near the threshold, even when 60 invasive cells were counted. Given that heterogeneity was ruled out, the discordant cases of the observers with lower performance were probably attributable to counting in non-cancer areas or in areas with excessive background, showing a wider range of *HER2*/CEP17 ratios. ISH analysis should start with scanning of the entire slide prior to counting, and compare with IHC to define the areas of potential *HER2* amplification. Furthermore, the absence of information on preanalytic conditions can jeopardize the execution of the ISH technique and create artefacts that impair proper measurement of the signals. Although chromosome 17 polysomy can cause impairment of ISH interpretation, it was not an issue in this study, given that no observer found such cases.

Unexpectedly, we found more discordant cases in core biopsies than in surgical specimens. Although counting additional cells eliminates this problem, we think that it is probably related to the higher quantity of tumour in surgical specimens, which allows for the identification of better scoring areas, with less

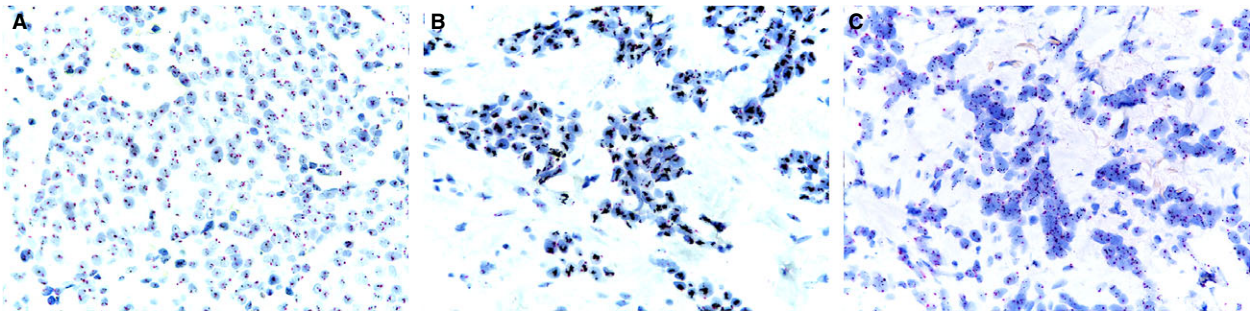

Figure 2. Examples of results of *HER2* detection with the silver *in-situ* hybridization technique. **A**, *HER2* negative for all observers. **B**, *HER2* positive for all observers. **C**, *HER2* discordancy among observers.

background and fewer overlapping cells. Previously, it has been shown that the identification of amplification status by SISH is very robust, as consensus between several observers cannot be reached in <2% of cases, although a consensus in discordant cases was not the aim in this study.[20]

As previously shown, the cases with amplification levels close to the thresholds are the most likely to be discordant.[23] For cases in which the *HER2*/CEP17 ratio lies between 1.80 and 2.20, the ASCO/CAP guidelines recommend that a different person counts an additional 20 invasive cells, whereas the updated UK guidelines recommend counting at least 60 invasive cells (preferably with a dual count by a second observer).[8,24] We agree with the UK guidelines recommendation, because 20 additional invasive cells evaluated by a different observer will not be sufficient to assess doubtful cases with an *HER2*/CEP17 ratio around 2.0, as we show in this study. Moreover, the similar variability of the *HER2*/CEP17 ratio within each evaluation, along with the divergent measurements between the first and second evaluations, suggests that the observers have the same error but different bias in different evaluations. We therefore recommend counting additional cells immediately than counting on a different day.

The UK guidelines for *HER2* assessment in BC recommend that, in cases with either clear amplification or an *HER2*/CEP17 ratio of <1.5, scoring of 20 tumour cells is sufficient.[24] Although our data support this recommendation for experienced observers, it is more practical to immediately count 40 tumour cells and calculate both the *HER2*/CEP17 ratio and the average *HER2* copy number than to count 20 cells, calculate these values, and then decide to count additional cells.

Training and experience in the interpretation of *HER2* ISH testing is essential. The UK guidelines recommend that laboratories perform at least 100 *HER2* ISH assays every year, and, when new personnel are being trained in the interpretation of *HER2* testing, observations of at least 100 ISH tests in parallel with an experienced scorer should be performed until a minimum concordance of 95% is achieved.[24] In our study, although both residents had the same level of experience after training, only one reached a concordance rate of at least 95% with the experienced observer, which underlines the importance of validation studies in this field.

The variability of both the *HER2*/CEP17 ratio and of the average *HER2* copy number in *HER2*-positive cases were always statistically higher than in *HER2*-negative cases, probably because of the different estimation of the number of *HER2* signals in clusters. Another explanation could be the genetic instability of *HER2* amplification already described between primary BC and metastatic lesions in *HER2*-positive cases, whereby the latter had a significant increase in *HER2* copy number.[25] In the same way, separate areas of *HER2*-positive tumours could, in fact, have different *HER2*/CEP17 ratios and *HER2* copy numbers, although this needs to be clarified in future studies. Finally, the lower variability of the *HER2*/CEP17 ratio than of the *HER2* copy number shows that the ratio value is probably more reproducible among observers, probably because the larger size of the CEP17 signal makes it easy to identify.[20,26] Although the ISH technique is the gold standard for identifying *HER2*-positive BC cases because it evaluates *HER2* amplification only in invasive cancer cells, it might not be the most accurate method for *HER2* quantification. One of the first studies investigating the effects of the level of *HER2* amplification showed that high-amplification tumours had a significantly higher rate of pathologically complete response to neoadjuvant treatment with trastuzumab than low-amplification tumours.[27] However, the same authors showed later that *HER2* amplification level was not

Table 6. Variability of *HER2*/CEP17 ratio in negative and positive cases with different cell counts

	Pathologist 1			Pathologist 2			Resident 1			Resident 2			
Cells	1st	2nd	1st–2nd	1st	2nd	1st–2nd	1st	2nd	1st–2nd	1st	2nd	1st–2nd	Result
20	0.23 (0.06–0.77)	0.24 (0.09–0.76)	0.36 (0.14–1.04)	0.27 (0.11–0.73)	0.34 (0.04–0.74)	0.47 (0.21–1.18)	0.30 (0.11–0.56)	0.26 (0.09–0.61)	0.38 (0.15–0.83)	0.34 (0.05–0.86)	0.28 (0.08–0.83)	0.54 (0.19–1.23)	N
	0.93 (0.39–3.86)	1.51 (0.11–3.30)	1.83 (0.82–5.03)	1.11 (0.37–3.50)	0.85 (0.34–5.31)	1.04 (0.52–5.31)	0.81 (0.17–2.38)	0.81 (0.16–2.73)	1.32 (0.24–4.54)	1.81 (0.46–2.60)	1.33 (0.24–3.91)	2.44 (1.09–4.87)	P
40	0.10 (0.01–0.31)	0.08 (0.00–0.30)	0.16 (0.04–0.78)	0.11 (0.01–0.32)	0.13 (0.01–0.33)	0.26 (0.08–1.10)	0.08 (0.02–0.43)	0.07 (0.00–0.44)	0.18 (0.03–0.67)	0.10 (0.01–0.40)	0.07 (0.01–0.32)	0.29 (0.05–1.01)	N
	0.46 (0.05–1.87)	0.30 (0.06–1.24)	0.92 (0.21–3.17)	0.33 (0.04–1.20)	0.33 (0.10–0.84)	0.54 (0.18–1.07)	0.25 (0.04–0.90)	0.22 (0.05–1.37)	0.53 (0.08–3.48)	0.45 (0.11–1.17)	0.38 (0.03–1.12)	1.25 (0.27–4.03)	P
60	NA	NA	0.06 (0.00–0.63)	NA	NA	0.15 (0.00–0.94)	NA	NA	0.08 (0.00–0.61)	NA	NA	0.19 (0.00–1.08)	N
	NA	NA	0.50 (0.00–2.48)	NA	NA	0.31 (0.07–2.50)	NA	NA	0.27 (0.01–3.03)	NA	NA	0.66 (0.04–3.24)	P

N, negative; NA, not applicable; P, positive.
Median (minimum–maximum).
Twenty cells versus 40 cells, and 40 cells versus 60 cells: Mann–Whitney *U*-test $P < 0.001$.
Negative versus positive: Mann–Whitney *U*-test $P < 0.001$.
1st versus 2nd: Mann–Whitney *U*-test $P > 0.05$.
1st versus 1st–2nd, and 2nd versus 1st–2nd: Mann–Whitney *U*-test $P < 0.05$.

Table 7. Variability of *HER2*/CEP17 ratio in discordant cases with different cell counts

	Pathologist 1			Pathologist 2			Resident 1			Resident 2		
Cells	1st	2nd	1st–2nd	1st	2nd	1st–2nd	1st	2nd	1st–2nd	1st	2nd	1st–2nd
20	0.46* (0.10–0.59)	0.55*† (0.24–0.79)	0.65*† (0.52–1.11)	0.79† (0.31–2.65)	0.84† (0.39–2.58)	1.01† (0.39–3.45)	0.59† (0.36–0.77)	0.46† (0.26–0.66)	0.96† (0.43–1.36)	0.93*† (0.22–3.63)	0.75*† (0.24–2.10)	1.13*† (0.52–6.76)
40	0.07 (0.07–0.07)	0.24† (0.17–0.30)	0.40† (0.30–0.69)	0.21† (0.15–0.53)	0.43† (0.33–0.53)	0.77† (0.18–2.01)	0.22 (0.22–0.22)	NA	0.42 (0.21–0.62)	0.34† (0.12–0.57)	0.17 (0.13–0.20)	0.77† (0.45–1.61)
60	NA	NA	NA	NA	NA	0.93*† (0.41–1.68)	NA	NA	0.42 (0.42–0.42)	NA	NA	0.63† (0.25–1.21)

NA, not applicable.
Median (minimum–maximum).
*Mann–Whitney *U*-test $P < 0.05$ (comparison with variability of positive cases).
†Mann–Whitney *U*-test $P < 0.05$ (comparison with variability of negative cases).

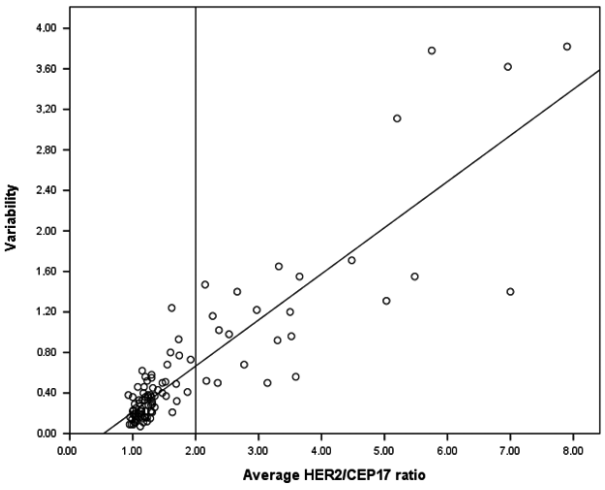

Figure 3. Relationship of the average of the *HER2*/CEP17 ratio with its variability when 60 invasive cells are counted.

correlated with either recurrence-free or overall survival in the same setting.[28] Moreover, the data from the Herceptin Adjuvant (HERA) trial were also used to compare the degree of *HER2* amplification with clinical outcome in *HER2*-positive BC (in both the untreated and trastuzumab-treated).[29] Both the *HER2*/CEP17 ratio and the *HER2* copy number were measured, and no significant effect on prognosis or benefit from trastuzumab was observed with different levels of *HER2* amplification. In contrast, it was recently reported that there was an increased risk of death from BC in the first 5 years after diagnosis in women with *HER2* copy numbers of ≥6, as well as of ≥4.0 and <6.0, irrespective of *HER2*/CEP17 ratio.[30] The conflicting clinical data suggest that the variability of the *HER2*/CEP17 ratio and the *HER2* copy number, depending on the number of cells counted, may determine the accuracy of the final result. Accordingly, to evaluate the putative clinical implications of the degree of *HER2* amplification, alternative methodology that quantifies *HER2* amplification more precisely must be used. Therefore, imaging analysis of ISH tests, counting several hundreds of cells more objectively, and *HER2* quantification by molecular techniques in separate areas of invasive carcinoma should be investigated to assess the variability of the results in *HER2*-positive BC cases.

In conclusion, we show that counting 20 cells in the *HER2* ISH test is not sufficient to obtain a reproducible result, and that the minimal cell number should be raised to at least 40, and preferably 60, invasive BC cells. Additionally, cases with amplification levels close to the threshold should have a count of at least 60 cells, and, if possible, a dual count from

an experienced observer. As far as we know, this is the first time that SISH results have been compared between multiple observers counting different numbers of invasive cancer cells.

Conflicts of interest

The authors declare no potential conflict of interest.

Author contributions

A. Polónia: designed the research study, analysed the data, and wrote the paper. A. Polónia, C. Eloy, J. Pinto, and A. Costa Braga: performed the research study, conducted *HER2* SISH interpretation, and retrieved the data. G. Oliveira: responsible for the performance of the SISH technique. F. Schmitt: wrote and critically revised the manuscript. All authors read and approved the final manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Minimal and maximal intraobserver correlation of the *HER2*/CEP17 ratio with different cell counts.

Table S2. Minimal and maximal intraobserver correlation of the average *HER2* copy number with different cell counts.

Table S3. Minimal and maximal interobserver correlation of the *HER2*/CEP17 ratio with different cell counts.

Table S4. Minimal and maximal interobserver correlation of the average *HER2* copy number with different cell counts.

Table S5. Minimum and maximum average *HER2* copy number in discordant cases with different cell counts.

Table S6. Variability of the average *HER2* copy number in negative and positive cases with different cell counts.

Table S7. Variability of the average *HER2* copy number in discordant cases with different cell counts.

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ORIGINAL ARTICLE

Characterization of HER2 gene amplification heterogeneity in invasive and in situ breast cancer using bright-field in situ hybridization

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Abstract The aims of this study were to evaluate and compare the HER2 gene amplification *status* in invasive and adjacent in situ breast carcinoma, using bright-field in situ hybridization, and to document the possible presence of HER2 genetic heterogeneity (HER2-GH) in both components. A cohort of 100 primary invasive carcinomas (IC) associated with carcinoma in situ (CIS) were evaluated for HER2 gene amplification by SISH according to the 2013 ASCO/CAP HER2 guideline. A second cohort of all the cases with HER2-GH since the introduction of the updated ASCO/CAP HER2 guideline was also characterized, and an evaluation of the HER2 gene amplification in the CIS component, if present, was also done. In the first cohort, the HER2 amplification in the IC was negative in 87% of the cases and positive in 13% of the cases, without the presence of HER2-GH. All the cases had an associated CIS with the same HER2 *status* as IC, with four cases of CIS presenting HER2-GH. In the CIS, we observed a significant relationship of HER2 gene amplification with high nuclear grade. In the four cases with HER2-GH in CIS, two cases presented HER2 gene amplification in the IC. The second cohort included 12 cases with HER2-GH in a total of 1243 IC cases (0.97%). Additionally, we identified two cases associated with non-amplified CIS. HER2-GH is a rare event in IC and can already be present in CIS, not being an important step in the acquisition of invasive features.

Keywords Breast cancer · HER2 · SISH · Invasive carcinoma · Carcinoma in situ · Genetic heterogeneity

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Introduction

Human epidermal growth factor receptor 2 (HER2) is amplified and/or overexpressed in about 15 to 20% of invasive breast cancer (BC), being associated with worse clinical outcome and predictive of benefit from HER2-targeted therapy [1–3]. The incidence rate of carcinoma in situ (CIS) of the breast, the immediate precursor of invasive carcinoma (IC), has stabilized since the beginning of the millennium in women older than 50 years, but continues to increase about 2% every year in younger women [4]. Several studies have shown that HER2 amplification can already be present in CIS and that frequently HER2 *status* is concordant with the invasive component [5, 6].

Heterogeneity has been noticed in almost all types of cancer, including BC, being related to several aspects of disease progression and clinical outcome [7]. The first recommendation regarding HER2 genetic heterogeneity (HER2-GH) was published in 2009 as an extension of the 2007 American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) HER2 guidelines after the acknowledgment that some tumors displayed intratumoral heterogeneity and such cases could originate discrepant results between immunohistochemistry (IHC) and in situ

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hybridization (ISH) analysis [8]. At that time, HER2-GH was defined as HER2 gene amplification in 5 to 50% of invasive cancer cells. Importantly, the definition was based on studies that did not include clinical outcome, being the first step to investigate the clinical significance of HER2-GH and the possible role of target therapy in this setting [9, 10]. Thereafter, numerous studies have shown that HER2-GH could be present in BC from 5 to 40% of the cases [11, 12]. Additionally, it was shown that HER2-GH was more frequent in cases near the threshold of positivity and that heterogeneity measured in individual cells is not informative of clonal heterogeneity within a tumor population [13].

Currently, the definition of HER2-GH has changed from individual cells to discrete population of tumor cells with HER2 gene amplification. According to the 2013 ASCO/CAP HER2 guideline, a tumor is considered HER2 positive if HER2 gene amplification is present in at least 10% of the total tumor cell population [14].

The aims of the present study are to compare the HER2 gene amplification *status* in invasive and adjacent in situ BC, using bright-field ISH, and to document the possible presence of heterogeneity in both components.

Materials and methods

Case selection

The cases included formalin-fixed, paraffin-embedded needle core biopsies (NCB) and surgical excision specimens (SES) referred to Ipatimup Diagnostics with an equivocal HER2 result (score of 2+) in IC by IHC for performance of an evaluation of HER2 amplification with bright-field ISH. There was no information regarding patient treatment.

The first cohort included 100 primary invasive BC cases associated with CIS retrieved from the archives from November 2015 to July 2016 to determine the concordance of HER2 gene amplification in both components. During this period, 347 cases with an equivocal HER2 result by IHC were evaluated for HER2 gene amplification. The cohort comprised 66 NCB and 34 SES, all diagnosed in women. The age of the patients ranged from 31 to 83 years old, with a median age at diagnosis of 54 years. The majority of the histological types were invasive carcinomas of no special type (NST), with 14% of the cases being classified as grade 1, 66% as grade 2, and 20% as grade 3 (Table 1).

The second cohort included all cases with HER2-GH (primary invasive or metastatic BC) since the introduction of the 2013 ASCO/CAP HER2 guideline (November 2013) to October 2016. An evaluation of HER2 gene amplification in the CIS component, if present, was also done. The cohort comprised 10 NCB and 2 SES with HER2-GH in a total of 1243 cases (0.97%), 11 of which were primary invasive BC and one lymph node metastasis. The age of the patients ranged from 42 to 74 years old, with a median age at diagnosis of 58 years, and two cases were diagnosed in men. All histological types but one were IC, NST, with eight cases being classified as grade 2 and four cases as grade 3 (Table 2).

Table 1 Characteristics of the first cohort

Procedure (NCB/SES)	66/34
Gender (female/male)	100/0
Age (mean ± SD)	54.68 ± 12.41
Invasive carcinoma	
Histological type	
Invasive carcinoma, NST	88
Lobular carcinoma	8
Micropapillary carcinoma	2
Mucinous carcinoma	1
Encapsulated papillary carcinoma	1
Histological grade	
Grade 1/grade 2/grade 3	14/66/20
HER2 gene amplification	
ISH negative/ISH positive	87/13
Carcinoma in situ	
Ductal	93
Lobular	8
Nuclear grade	
Low/intermediate/high	3/42/56
Necrosis (absent/present)	58/43
Microcalcifications (absent/present)	73/28

NCB needle core biopsy, *SES* surgical excision specimen, *SD* standard deviation, *NST* no special type

All cases were reviewed for histological type and grade (Nottingham Histologic Score) in the IC. The characterization of the CIS included nuclear grade, the presence of necrosis, and microcalcifications.

This study has been performed in accordance with the national regulative law for the handling of biological specimens from tumor banks, being the samples exclusively available for research purposes in retrospective studies, as well as under the international Helsinki declaration.

Silver in situ hybridization

SISH technique was performed on 3-μm-thick sections in one block of each case with dual-hapten, dual-color ISH. The dual-probe assay (INFORM HER2 Dual ISH DNA Probe Cocktail Assay; Ventana Medical Systems, Inc., Tucson, AZ, USA), which is Food and Drug Administration-approved, contains an HER2 locus-specific probe (black signal) and a control probe specific for the centromere of chromosome 17 (centromere enumeration probe-CEP17, red signal), which allows detection of HER2 gene amplification by light

Table 2 Characteristics of the second cohort

Procedure (NCB/SES)	10/2
Gender (female/male)	10/2
Age (mean ± SD)	59.67 ± 10.92
Invasive carcinoma	
Histological type	
Invasive carcinoma, NST	11
Lobular carcinoma	1
Histologic grade	
Grade 1/grade 2/grade 3	0/8/4
Carcinoma in situ	
Ductal	2
Nuclear grade	
Low/intermediate/high	0/1/1
Necrosis (absent/present)	1/1
Microcalcifications (absent/present)	2/0

NCB needle core biopsy, *SES* surgical excision specimen, SD standard deviation, *NST* no special type

microscopy. The entire procedure was carried out on an automated staining system (Ventana BenchMark XT Staining System; Ventana Medical Systems, Inc., Tucson, AZ, USA) according to the manufacturer's instructions. Appropriated positive and negative controls were used in every set of slides.

SISH interpretation

The evaluation of the samples included scoring of at least 20 nuclei, in two different areas, recording the number of HER2 and CEP17 signals. Corresponding hematoxylin and eosin (H&E) staining was used for the identification of the invasive and in situ components of the tumor, and only cells with a minimum of one copy of HER2 and CEP17 each were scored. The number of HER2 signals was estimated in clusters, except for doublets which counted as a single signal. The samples were classified by a pathologist (AP) according to the 2013 ASCO/CAP ISH criteria for HER2 gene amplification: positive when the HER2/CEP17 ratio is ≥2.0 or <2.0 and the average HER2 copy number is ≥6.0 signals *per* cell; equivocal when the HER2/CEP17 ratio is <2.0 and the average HER2 copy number is ≥4.0 and <6.0 signals *per* cell; and negative when the HER2/CEP17 ratio is <2.0 and the average HER2 copy number is <4.0 signals *per* cell [14].

HER2-GH is defined as tumors with discrete population of tumor cells with different HER2 gene *status* [14]. The proportion of amplified areas was quantified by measuring the number of fields (power field of 200×) with HER2 gene amplification divided by the number of fields of invasive or in situ carcinoma.

Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 24.0 for Windows. The Pearson's Chi-squared (χ^2) test (or the Fisher's exact test, if appropriate) was used for comparison of qualitative variables and the Mann-Whitney *U* (MWU) test, the *t* test, and Pearson's correlation coefficient (PCC) were used for comparison of quantitative variables. The level of significance was set at $p < 0.05$.

Results

In the first cohort, the HER2 amplification in the IC was negative in 87% of the cases and positive in 13% of the cases, without the presence of HER2-GH. All the cases had an associated CIS with the same HER2 *status* than IC, with four cases of CIS presenting HER2-GH. Because one case presented both lobular and ductal CIS, we characterized 101 types of CIS, where 92.1% were ductal CIS (DCIS) and 2.9% of the cases were classified as low grade, 41.6% as intermediate grade, and 55.5% as high grade. We also observed necrosis in 42.6% and microcalcifications in 27.7% (Table 1).

HER2 amplification in the IC was not related with the procedure, the age of the patients or the histological grade. However, in the CIS, we observed a significant relationship of HER2 amplification with high nuclear grade (18.9 vs 4.4%; $p = 0.030$), without an association with the remaining characteristics (Table 3).

The distribution of HER2/CEP17 ratio and average of HER2 and CEP17 copy number *per* cell were not statistically different between the IC and CIS (Table S1). Additionally, we observed a high correlation of HER2/CEP17 ratio and average HER2 copy number *per* cell between IC and CIS (PCC = 0.981; $p < 0.001$ and PCC = 0.929; $p < 0.001$, respectively) (Fig. 1 and S1).

In the four cases with HER2-GH in CIS, only one case was identified in NCB and two cases presented HER2 gene amplification in the IC (Figs. 2 and 3). The proportion of cells with HER2 gene amplification in the CIS varied between 30 and 60% of the total CIS represented in the sample, being all high-grade DCIS (for details, see Table 4 and S2).

In the second cohort, the proportion of cells with HER2 amplification varied between 1 and 50% of the total tumor cell population represented in the sample. In the negative component, HER2/CEP17 ratio varied between 1.00 and 1.52, and in the positive component between 2.06 and 9.17 (Table S3).

The primary IC cases had similar morphological features in the amplified and non-amplified components. The lymph node metastasis case represented a primary lobular carcinoma of the breast with an equivocal HER2 result (score of 2+) by IHC and no HER2 gene amplification by SISH (case 2). The

Table 3 HER2 amplification in invasive and in situ carcinoma

Invasive carcinoma	Negative	Positive	*p*
Procedure (NCB/SES)	58/29	8/5	0.759[a]
Age (mean ± SD)	54.52 ± 12.86	55.69 ± 9.29	0.753[b]
Histological grade (grade 1–2/grade 3)	72/15	8/5	0.129[a]
Carcinoma in situ			
Ductal/lobular	78/8	12/0	0.590[a]
Nuclear grade (low-intermediate/high)	43/43	2/10	*0.030*[c]
Necrosis (absent/present)	52/34	5/7	0.216[c]
Microcalcifications (absent/present)	65/21	6/6	0.085[a]

NCB needle core biopsy, *SES* surgical excision specimen, *SD* standard deviation

[a] Fisher's exact test

[b] *t* test

[c] Pearson's Chi-squared test

metastasis showed two types of neoplastic cells, one similar with the primary lesion and another one with more pleomorphic nuclei. After additional IHC in the metastasis, it was found that the latter areas showed strong and complete membranous staining in 40% of the cells (score of 3+) that were confirmed by SISH as HER2 amplified (Fig. 4).

Additionally, in the primary IC cases, we identified two cases associated with non-amplified DCIS (cases 11 and 12—Table 5 and S4). Case 12 was classified as equivocal by IHC (score of 2+) because it showed strong and complete membranous staining in small groups of tumor cells, along with scattered single cells, representing less than 10% of the total tumor cell population in the sample. After SISH analysis, the same component presented HER2 gene amplification, consequently being classified as HER2 ISH negative (Fig. 5).

Discussion

The aim of the present study was to compare the amplification *status* of HER2 gene between IC and CIS and search for HER2-GH in both components. Our results show that in all the cases, we observed the same HER2 *status* in both IC and adjacent CIS, according to previous studies [15, 16]. Although, in this work, we only consider cases with an equivocal HER2 result by IHC that can bias the results, these are the cases that require reflex testing making the issue of HER2-GH in ISH evaluation more important in this setting.

Bright-field ISH allows to better correlate tissue morphology and HER2 gene *status*, clearly identifying HER2-GH in IC and CIS [17]. In CIS, HER2-GH was recognized more frequently in SES, in which more tissue is available for

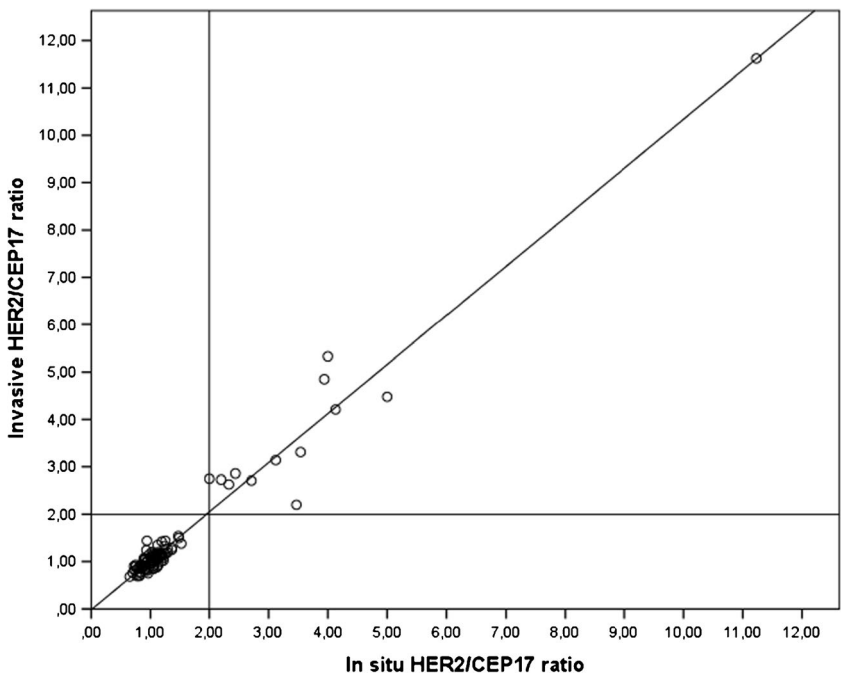

Fig. 1 Relationship between HER2/CEP17 ratio of in situ and invasive carcinoma

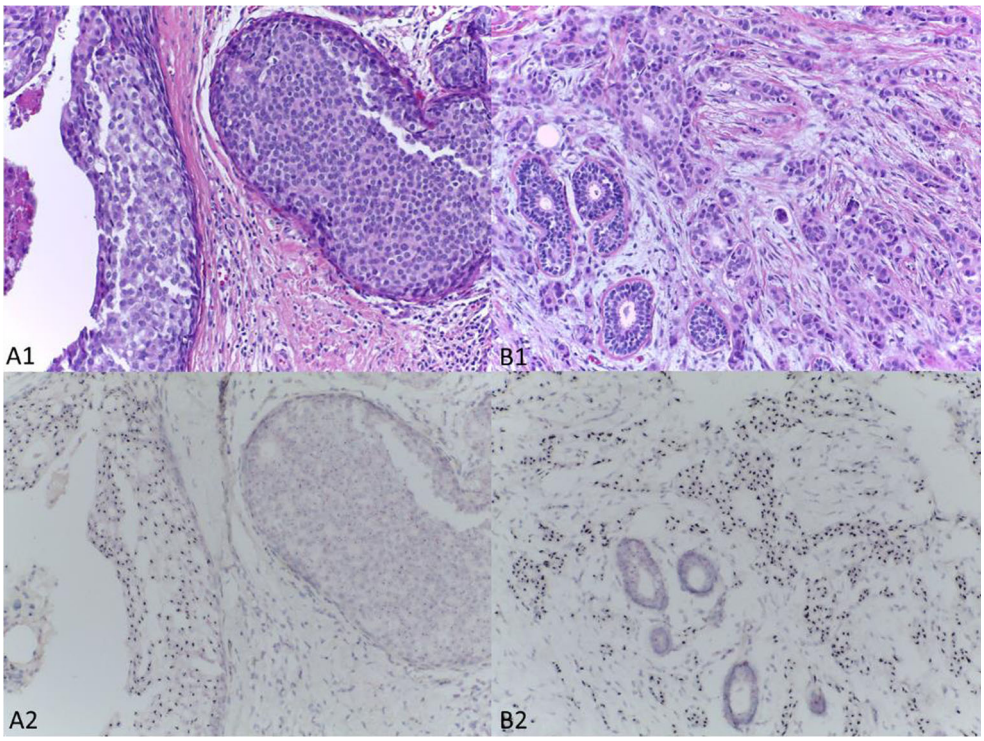

Fig. 2 Carcinoma in situ with HER2 genetic heterogeneity associated with HER2-positive invasive carcinoma. Case 1 (1st cohort): **a1** DCIS, H&E 200×; **a2** amplified DCIS (*left*) and non-amplified DICS (*right*), SISH 200×; **b1** invasive carcinoma, NST, H&E 200×; **b2** amplified invasive carcinoma, NST, SISH 200×

evaluation. Interestingly, in these cases, only half of IC presented HER2 gene amplification, confirming that this amplification is not relevant for the transition from CIS to IC. In the literature, it has been documented that in HER2-positive cases, a significant increase occurs in HER2 copy number between primary BC and metastatic lesions [18]. The high correlation of HER2/CEP17 ratio and HER2 copy number between IC and CIS suggests that the described genetic instability of HER2 gene amplification is only present in metastatic stages.

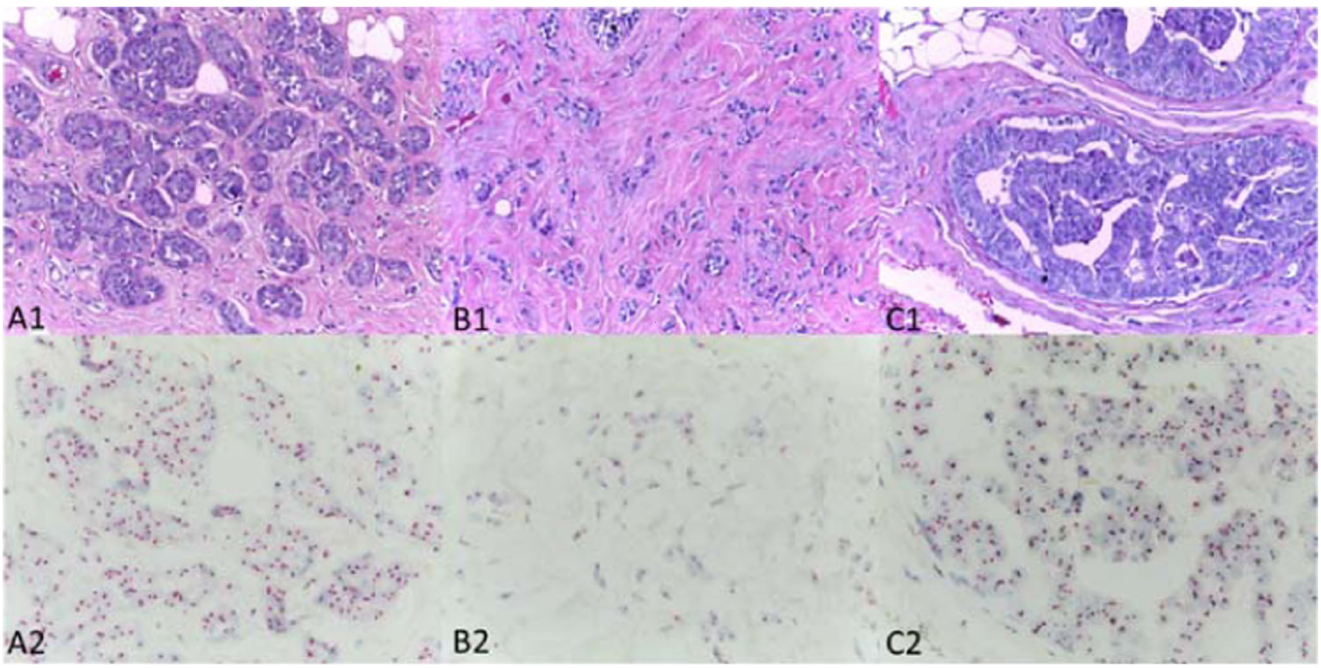

Fig. 3 Carcinoma in situ with HER2 genetic heterogeneity associated with HER2-negative invasive carcinoma. Case 4 (1st cohort): **a1** LCIS, H&E 200×; **a2** non-amplified LCIS, SISH 400×; **b1** invasive lobular carcinoma, H&E 200×; **b2** non-amplified invasive lobular carcinoma, SISH 400×; **c1** DCIS, H&E 200×; **c2** amplified DCIS, SISH, 400×

Table 4 Cases with HER2 amplification discordance between invasive carcinoma and carcinoma in situ

Case	Gender	Age	Procedure	IC	Histologic grade	HER2 status	CIS	Nuclear grade	HER2 status	Proportion
1	F	44	SES	NST	2	A	ductal	high	A	40%
									NA	60%
2	F	53	SES	NST	3	A	ductal	high	A	60%
									NA	40%
3	F	48	NCB	NST	3	NA	ductal	high	NA	40%
									A	60%
4	F	78	SES	Lobular	1	NA	lobular	low	NA	70%
							ductal	high	A	30%

F female, *SES* surgical excision specimen, *NCB* needle core biopsy, *NST* no special type, *A* amplified, *NA* not amplified

In our study, HER2 gene amplification in IC was not associated with histological grade, contrary to what has been published [6, 19]. Current evidence shows that histological grading of NCB can only be concordant with SES in about 75% of the cases [20, 21]. Most of the discordant cases are upgraded in the SES, generally due to an underscored of the mitotic frequency on NCB [21, 22]. Although our first cohort included a slight increase of grade 2 tumors compared with expected values in the literature, it might be the result of the large number of NCB, which can underestimate the histological grade and compromise the statistical relationship with HER2 gene amplification [20]. Nevertheless, HER2 gene amplification in CIS was significantly associated with high nuclear grade, as previously documented [6, 19].

Before the introduction of the first definition of HER2-GH, intratumoral heterogeneity was applied to discrete population of cells and reported as a rare event [23, 24]. Recently, we also showed the presence of HER2-GH in IC, according to the updated ASCO/CAP HER2 guideline, to be extremely infrequent [25]. In the present work, we identified it in about 1% of the cases, including cases of male patients.

HER2-GH in the IC was observed more often in NCB, because most evaluations are performed by this procedure, which represent the first biological material on which the hormone receptors (HR) and HER2 markers should be first determined [14, 26]. The predominant histologic type was invasive carcinomas, NST, with no cases classified as grade 1, as previously noticed [23]. Although we had no information

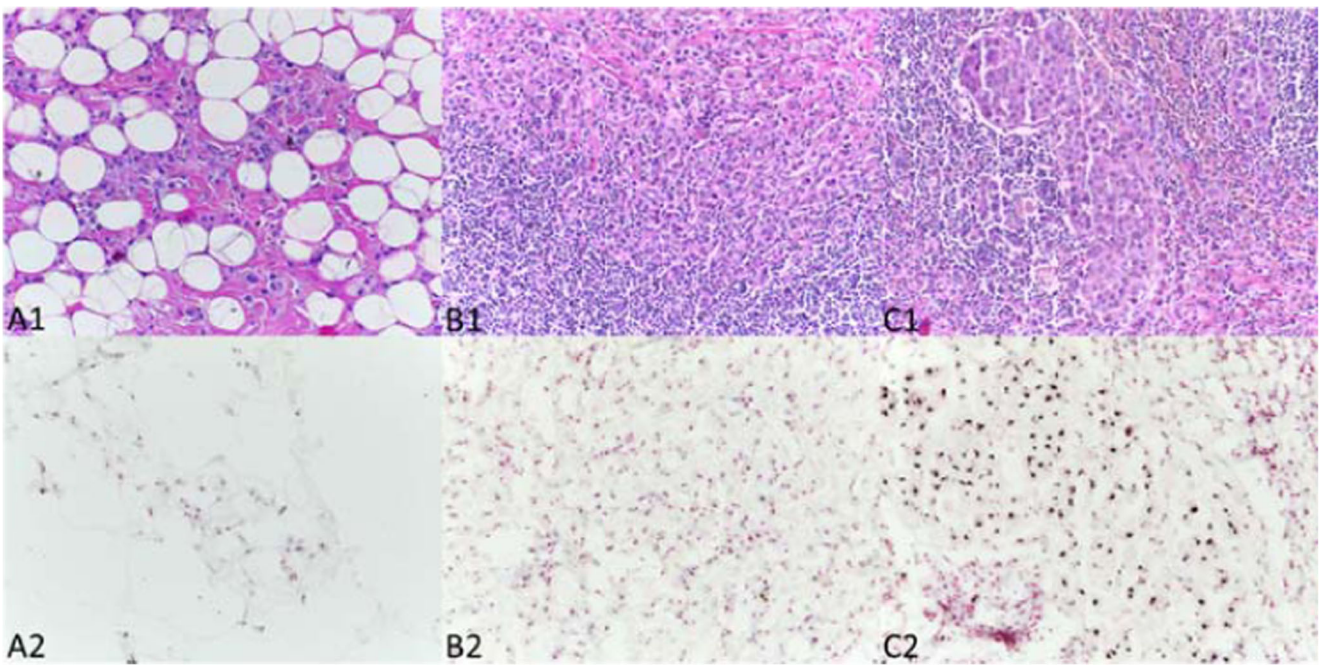

Fig. 4 HER2-negative invasive carcinoma associated with HER2 genetic heterogeneity in the lymph node metastasis**.** Case 2 (2nd cohort): **a1** invasive lobular carcinoma, H&E 200×; **a2** non-amplified invasive lobular carcinoma, SISH, 400×; **b1, c1** same lymph node metastasis, H&E 200×; **b2** non-amplified area in lymph node metastasis; **c2** amplified area in lymph node metastasis, SISH 400×

Table 5 Cases with HER2 genetic heterogeneity in invasive carcinoma associated with carcinoma in situ

Case	Gender	Age	Procedure	IC	Histologic grade	HER2 status	Proportion	CIS	Nuclear grade	HER2 status
11	F	52	NCB	NST	3	A	25%			
						NA	75%	ductal	high	NA
12	M	73	NCB	NST	2	NA	99%	ductal	intermediate	NA
						A	1%			

F female, *M* male, *NCB* needle core biopsy, *IC* invasive carcinoma, *NST* no special type, *A* amplified, *NA* not amplified, *CIS* carcinoma in situ

regarding previous treatment, the fact that most IC cases with HER2-GH were found in NCB shows us that this rare event can occur in patients without previous treatment.

All primary invasive BC cases presented similar histological characteristics in the amplified and non-amplified areas, with HER2 gene amplification in a minor component, supporting the idea that most cases develop in a single tumor that acquired HER2 gene amplification during tumor progression. Even though molecular analysis has shown that cases with HER2-GH in the IC can be the result of two distinct tumors (also known as collision tumors), most cases appear to be clonally related resulting from clonal divergence from a single tumor, as previously shown [27, 28]. However, it remains to be shown if the same process can occur in CIS.

In case 2 (second cohort), the HER2-GH was found in the lymph node metastasis rather than the primary tumor. This can be the result of tumor evolution in the lymph node metastasis or, eventually, the representation of an independent tumor that was not identified in the SES. Nevertheless, this case illustrates the importance of pathologists in selecting tumor areas with less differentiation and higher nuclear pleomorphism, either in the primary IC or in the lymph node metastases, which most likely are going to be HER2 amplified. Additionally, according to the 2013 ASCO/CAP guideline, the HER2 test should also be repeated if results are discordant with histopathologic findings [14].

Although it has been previously reported that HRs and HER2 conversion by IHC can occur between primary and metastatic lesions, the former is much more frequent (preferentially from HR-positivity in the primary tumors to HR-negativity in the metastasis) [18, 29]. The conversion phenomenon could be explained by HER2-GH in the primary tumor, which can be more frequently found if more than one block is tested [30, 31]. In this study, we were restricted to the analysis of only one block, which can underestimate the prevalence of HER2-GH. Additionally, we also not considered

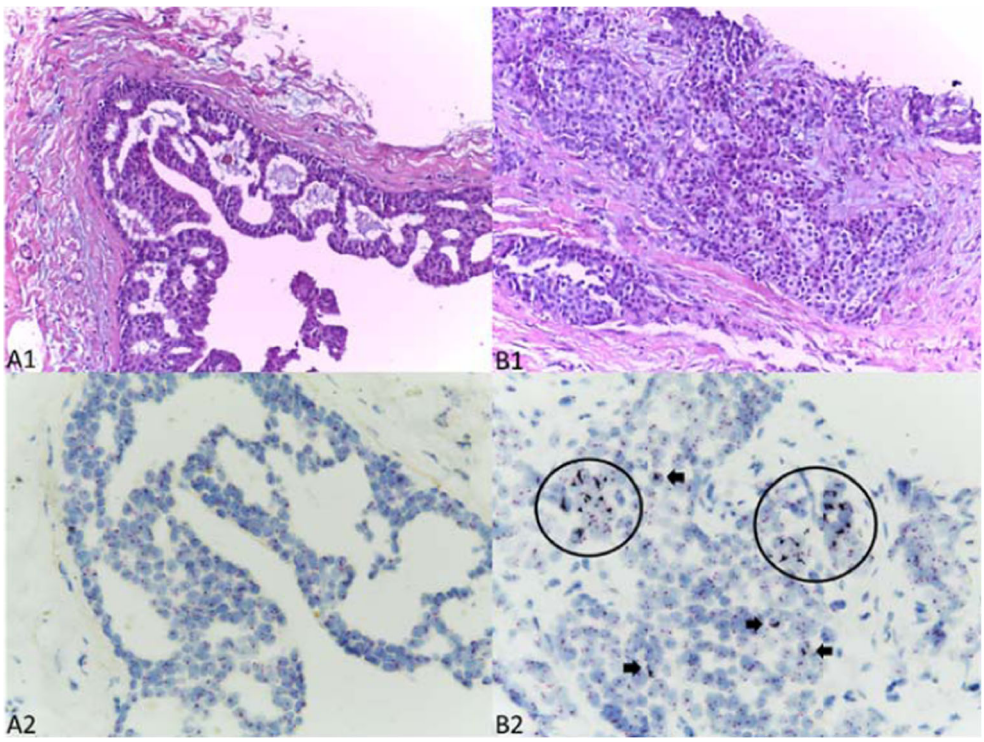

Fig. 5 Invasive carcinoma with HER2 genetic heterogeneity associated with HER2-negative carcinoma in situ. Case 12 (2nd cohort): **a1** DCIS, H&E 200×; **a2** non-amplified DCIS, SISH 400×; **b1** invasive carcinoma, NST, H&E 200×; **b2** HER2 genetic heterogeneity in invasive carcinoma (HER2 gene amplification in small groups of tumor cells (*circle areas*) along with scattered single cells (*arrows*))

cases with HER2-GH by IHC (score 3+ in >10 and <100%). Interestingly, it has also been shown that patients with HER2-GH have worse outcome compared to patients with homogeneous amplified or non-amplified HER2 gene, suggesting that mixed tumors behave more aggressively [32, 33].

Furthermore, it has been described that the majority of ICs with HER2-GH have non-amplified DCIS, consistent with our study, again supporting the idea that IC originates from CIS and that HER2 gene amplification can also be acquired in later stages [23]. Cases with HER2-GH in the IC associated with amplified DCIS probably represent distinct tumors, given the fact that the loss of the HER2 gene amplification is an unlikely event.

Finally, regarding case 12 (second cohort), which presented HER2 gene amplification in less than 10% of the total tumor cell population, a comment was made in the report recommending repetition of HER2 test by IHC in the SES to find and accurately quantify the HER2 positive component. Moreover, focally amplified small populations can be overlooked and IHC should be used to guide ISH analysis, searching for areas of potential amplification [14]. All cases exhibiting HER2-GH on NCB by ISH should have HER2 test repeated on the SES, according to the updated United Kingdom guidelines [34]. Additionally, clinical trials have not so far been based on the new 10% cut-off for ISH as provided by the 2013 ASCO/CAP guideline [35–39]. In fact, the presence of HER2 gene amplification was enough for inclusion of patients to HER2-targeted therapy, irrespective of the proportion of amplified cells. Fortunately, the rare presence of HER2-GH probably did not influence the clinical results given that any random group of cells evaluated will represent, most of the times, the whole tumor [40]. However, it remains to be demonstrated what is the minimal proportion of amplified tumor cell population that achieves clinical response to HER2-targeted therapy.

In conclusion, we show that HER2-GH is a rare event in IC and can already be present in CIS, not being an important step in the acquisition of invasive features. As far as we know, this is the first time that HER2-GH, according to the updated ASCO/CAP HER2 guideline, has been evaluated in both IC and adjacent CIS using bright-field ISH. Although intratumoral heterogeneity of HER2 gene amplification can have clinical significance, not only affecting the selection of patients but also explaining some of the variability of the response to targeted therapy, this is a rare event in breast cancer cases.

Authors's contributions AP: designed the research study, conducted HER2 SISH interpretation, retrieved and analyzed the data, and wrote the manuscript. GO: performed the laboratory work. FS: designed the research study and critically revised the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards This study has been performed in accordance with the national regulative law for the handling of biological specimens from tumor banks, being the samples exclusively available for research purposes in retrospective studies, as well as under the international Helsinki declaration.

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Conflict of interest The authors declare that they have no conflict of interest.

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Prognostic value of stromal tumour infiltrating lymphocytes and programmed cell death-ligand 1 expression in breast cancer

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ABSTRACT

Aim The present work aims to evaluate the presence of stromal tumour-infiltrating lymphocytes (TILs) and programmed cell death-ligand 1 (PDL1) expression in breast carcinomas and their correlation with available clinicopathological features.

Methods Two independent series of invasive breast cancer (IBC), one including ductal carcinoma in situ (DCIS) pair-matched cases, were selected, and quantification of TILs was accomplished in each case. Immunohistochemistry was also performed to evaluate the expression of PDL1.

Results In both cohorts evaluated, increased stromal TILs and PDL1 expression were present in about 10% of IBCs, being significantly associated with each other and both with grade 3 and triple-negative subtype. We observed a similar distribution of stromal TILs and PDL1 expression between DCIS and IBC. Finally, we observed that increased stromal TILs and PDL1 expression were significantly associated with cancer stem cell (CSC) markers, basal cell markers and vimentin expression. Interestingly, in IBC cases with vimentin expression, increased stromal TILs, as well as decreased PDL1 expression, disclosed a better clinical outcome, independently of the main classical BC prognostic factors.

Conclusions We have confirmed the association of stromal TILs and PDL1 expression with aggressive forms of BC and that both are already found in in situ stages. We also showed that stromal TILs and PDL1 expression are associated with clinical outcome in cases enriched for a mesenchymal immunophenotype. We describe for the first time a close relationship between CSC markers and PDL1 expression.

INTRODUCTION

In the Western world, breast cancer (BC) is the most frequently diagnosed malignancy among women, representing about one-third of all new cancer cases and the second leading cause of cancer death after lung cancer.[1] BC development and progression is dependent on a complex system of different factors, including genetic and epigenetic alterations, and on factors from the tumour microenvironment, such as stromal and immune cells.[2] In fact, in recent years, numerous studies have focused on the presence and function of the host immune system and its relationship with tumour progression in a variety of solid tumours, including BC, showing that spontaneous intratumoural lymphocytic infiltrate is related to patient prognosis.[3–9]

Although the BC's inflammatory infiltrate has been studied for several decades with conflicting results, large cohorts have recently shown an association between the presence of tumour-infiltrating lymphocytes (TILs) with improved prognosis and better response to neoadjuvant chemotherapy, regardless of the absence of information of its specific immune cells.[10 11] In triple-negative (TN) BC, for instance, the presence of stromal TILs in tumour tissue at diagnosis associates with better patient outcome after adjuvant anthracycline-based chemotherapy.[12] Similarly, in human epidermal growth factor receptor 2 (HER2)-positive BC, the number of TILs in tumour tissue associates with a better response to trastuzumab treatment.[13]

However, in contrast to the presumed protective effect of TILs in tumour tissue, it has been shown that immune cells can cause the acquisition of stem cell properties by tumour cells, as well as a more pronounced mesenchymal phenotype, which are features related with a worse patient prognosis.[14] Moreover, it was also shown that tumour cells express antigens that should be recognised by patient's immune system, although most of the time the immunological response is unable to eliminate the cancer cells. Currently, many efforts have been made to identify molecular mechanisms that enable tumour cells to escape from the host immune system.[2] An example of tumour escape from immunosurveillance is the expression of PDL1 (programmed cell death-ligand 1) by neoplastic cells, which is a cell surface glycoprotein that conveys an inhibitory signal to T lymphocytes, through the interaction with its receptor PD1 (programmed cell death protein 1). This specific binding leads to a decrease in cytokine production and an increase of T lymphocyte apoptosis, which protect tumour cells from elimination.[15–18] Accordingly, the inhibition of this inhibitory signal by specific monoclonal antibodies, against either PDL1 or PD1, has been shown to promote tumour cell death induced by the host immune system in many cancer models.[17 19]

Based on these data, the expression of PDL1 is already being evaluated in several solid tumours, such as melanoma, non-small cell lung carcinoma and renal cell carcinoma, since it brings additional information to patient prognosis and to the selection of immunotherapy currently available targeting these molecules specifically.[20] In line with these studies, the aim of the present work was to evaluate the relationship between the presence of stromal TILs and PDL1 expression with clinicopathological features in two independent BC series. The

association with disease-free-survival (DFS) and overall survival (OS) was also evaluated.

MATERIALS AND METHODS

Tumour samples

Two independent series of BC cases were studied, both with clinical and pathological characterisation performed by our group, previously described and structured in tissue microarrays (TMAs). The first cohort includes 440 primary and sporadic invasive ductal carcinomas retrieved from the Pathology Department, Hospital Xeral-Cíes, Vigo, Spain, diagnosed between 1978 and 1992, with a median age of 60.0 years (from 28 to 92 years) and a median follow-up time of 120 months (1–120 months).[21] Several parameters were extracted from the group database, including age, tumour size, histological grade, lymph node *status*, oestrogen receptor (ER), progesterone receptor (PgR), HER2 and Ki67 expression, as well as the expression of basal cell markers (CK5, CK14, epidermal growth factor receptor (EGFR) and P-cadherin), cancer stem cell (CSC) markers (CD24, CD44, CD49f and ALDH1) and the epithelial–mesenchymal transition (EMT) markers E-cadherin and vimentin. Data concerning molecular subtype, DFS and OS were also available. The main features are detailed in online supplementary table S1.

The second cohort includes 94 primary in situ and invasive BC (IBC) cases, including 32 pair-matched cases, collected from the Pathology Institute of Araçatuba, São Paulo, Brazil, diagnosed between 1996 and 2006, with a median age of 55.0 years (from 32 to 96 years).[22] The data retrieved from the database include age, histological grade, lymph node *status*, hormone receptor (ER and PgR), HER2, Ki67 and molecular subtype. The main features are detailed in online supplementary table S2.

This study has been performed in accordance with the national regulative law for the handling of biological specimens from tumour banks, the samples being exclusively available for research purposes in retrospective studies, as well as under the international Helsinki declaration.

Quantification of TILs

Histopathological analysis of the lymphocytic infiltrate was performed according to the guidelines for clinical and research practice.[23] Briefly, mononuclear cells, including lymphocytes and plasma cells (granulocytes excluded), were quantified in the stromal compartment as a continuous variable of 10% increment, within the borders of the invasive tumour, using visual assessment of H&E-stained sections. Thresholds were then used to categorise the continuous variable (absent—absence of TILs; slight—TILs up to 30%; moderate—TILs between 30% and 60%; marked—TILs in more than 60%). In ductal carcinoma in situ (DCIS), the lymphocytic infiltrate was quantified around the lesion using the same classification.

PDL1 immunohistochemistry

Immunohistochemical staining for PDL1 was performed in 2–3 μm sections from TMAs, using a rabbit monoclonal antibody (clone SP142; 1:60 dilution; Spring Bioscience, Pleasanton, California, USA). The assay was carried out on an automated immunostaining system (Ventana BenchMark XT Staining System), using the OptiView DAB IHC Detection Kit (Ventana Medical Systems, Tucson, Arizona, USA) according to manufacturer's instructions. Positive (human placenta) and negative staining controls were performed in parallel with paraffin sections. Positivity was defined as membranous and cytoplasmic staining ≥1% in both tumour cells and stromal TILs.[24]

Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS), V.21.0, for Windows. Pearson's χ^2 test (or Fisher's exact test, when appropriate) was used for comparison of qualitative variables, and the t-test or the Mann-Whitney U test for quantitative variables.

Survival rate curves were calculated according to the Kaplan-Meier method and compared by the log-rank test. DFS time was defined as the interval between diagnosis and BC recurrence or metastasis, whereas OS time was defined as the interval between diagnosis and BC-related death or between diagnosis and the last follow-up time for surviving patients. Multivariate survival analyses were based on the Cox proportional hazard regression model. The level of significance was set at $p<0.05$.

RESULTS

Association of stromal TILs and PDL1 expression with clinicopathological characteristics

The quantification of stromal TILs in the first cohort ranged between 0% and 80% (median of 10%), with a minority of cases having more than 30% (moderate to marked—9.2%) (table 1 and figure 1). However, the presence of moderate to marked stromal TILs was significantly increased in G3 (13.6%; $p<0.001$), in ER-negative cases (16.2%; $p<0.001$), in TNBC subtype (19.0%; $p<0.001$) and in cases with high expression of Ki67 (20.0%; $p=0.019$) (table 2). A significant association between moderate and marked stromal TILs with all the evaluated basal cell markers was also observed (CK5—22.4%; $p<0.001$/CK14—28.6%; $p=0.005$/EGFR—25.0%; $p=0.021$/P-cadherin—15.1%; $p=0.007$) (table 3). Additionally, we still found a significant association between stromal TILs and the expression of the CSC marker ALDH1 (33.3%; $p=0.014$), as well as with the expression of vimentin (17.2%; $p=0.008$) (table 3).

The expression of PDL1 was mainly found at the cell membrane (in both tumour cells and stromal TILs) and only present in 6.4% of the cases (table 1 and figure 1). Although PDL1 expression was not observed in normal breast tissue, it was significantly associated to G3 (10.1%; $p=0.002$) and ER-negative carcinomas (11.1%; $p=0.001$), as well as with the TNBC subtype (16.2%; $p<0.001$) and with cases with high expression of Ki67 (16.0%; $p=0.002$) (table 2). We also observed a significant direct association between PDL1 expression and all the evaluated basal cell markers (CK5—18.6%; $p<0.001$/CK14—18.2%; $p=0.031$/EGFR—20.0%; $p=0.022$/P-cadherin—13.1%; $p<0.001$), all the evaluated CSC markers ($CD44^+/CD24^{-/low}$—8.4%; $p=0.044$/CD49f—15.2%; $p=0.011$/ALDH1–23.1%; $p=0.033$) and with the expression of vimentin (12.3%; $p=0.036$) (table 3).

Stromal TILs and PDL1 expression were still significantly associated between each other (64.3%; $p<0.001$) (table 4).

Table 1 Stromal TILs and PDL1 expression in BC cases from the first cohort

TILs	
Absent/slight	178 (41.0%)/216 (49.8%)
Moderate/marked	36 (8.3%)/4 (0.9%)
PDL1 expression	
Negative	407 (93.6%)
Positive	28 (6.4%)

BC, breast cancer; PDL1, programmed cell death-ligand 1; TILs, tumour-infiltrating lymphocytes.

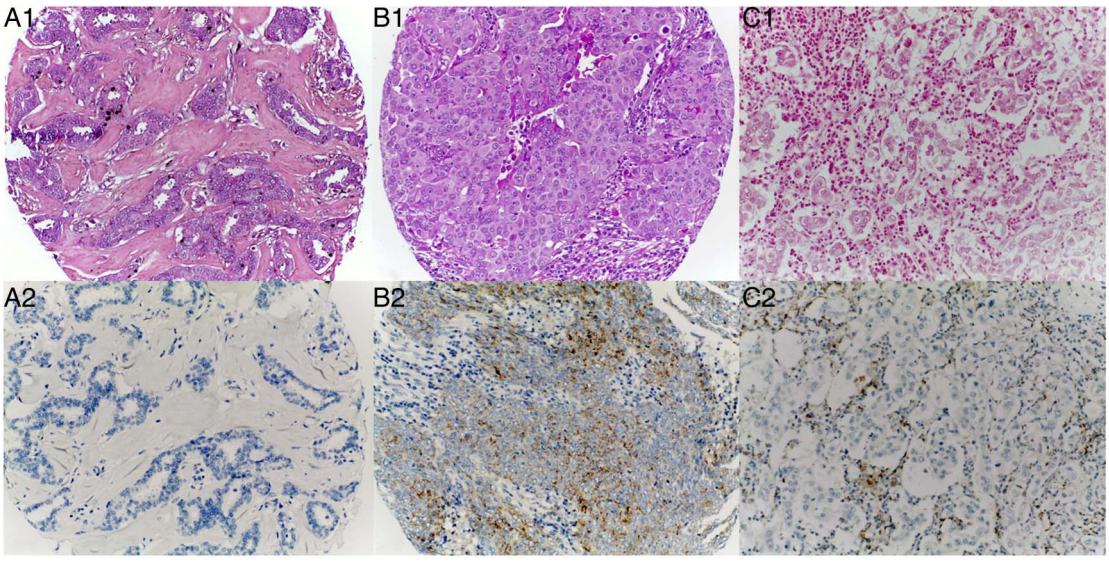

Figure 1 Stromal tumour-infiltrating lymphocytes (TILs) and programmed cell death-ligand 1 (PDL1) expression in invasive breast cancer (IBC) (200×). (A) 1—BC without stromal TILs (H&E); 2—PDL1 negative expression. (B) 1—BC with increased stromal TILs (H&E); 2—PDL1 expression in cancer cells. (C) 1—BC with increased stromal TILs (H&E); 2—PDL1 expression in stromal TILs.

Table 2 Association between clinicopathological features with stromal TILs and PDL1 expression in invasive breast cancer (first cohort)

	Stromal TILs			PDL1 expression		
Clinicopathological features	**Absent/slight**	**Moderate/marked**	**p Value**	**Negative**	**Positive**	**p Value**
Age (average±SD)	59.40±13.11	54.88±13.48	0.052*	58.89±13.27	60.26±11.57	0.763*
Tumour size			0.397†			0.069†
≤2 cm	87 (94.6%)	5 (5.4%)		92 (98.9%)	1 (1.1%)	
<2 cm to ≤5 cm	191 (92.3%)	16 (7.7%)		192 (92.3%)	16 (7.7%)	
>5 cm	45 (88.2%)	6 (11.8%)		48 (92.3%)	4 (7.7%)	
Histological grade			**<0.001**†			**0.002**†
G1	71 (98.6%)	1 (1.4%)		72 (100%)	0 (0%)	
G2	105 (97.2%)	3 (2.8%)		106 (97.3%)	3 (2.7%)	
G3	171 (86.4%)	27 (13.6%)		179 (89.9%)	20 (10.1%)	
Lymph node			0.164†			*0.612*†
Negative	126 (88.7%)	16 (11.3%)		133 (93.0%)	10 (7.0%)	
Positive	164 (93.2%)	12 (6.8%)		168 (94.4%)	10 (5.6%)	
ER			**<0.001**†			**0.001**†
Positive	246 (95.4%)	12 (4.6%)		253 (96.9%)	8 (3.1%)	
Negative	114 (83.8%)	22 (16.2%)		120 (88.9%)	15 (11.1%)	
PgR			0.874†			0.080†
Positive	175 (91.6%)	16 (8.4%)		184 (96.3%)	7 (3.7%)	
Negative	186 (91.2%)	18 (8.8%)		190 (92.2%)	16 (7.8%)	
HER2			0.308‡			0.757‡
Negative	308 (91.9%)	27 (8.1%)		317 (94.6%)	18 (5.4%)	
Positive	50 (87.7%)	7 (12.3%)		55 (93.2%)	4 (6.8%)	
Ki67			**0.019***			**0.002***
<14%	342 (92.2%)	29 (7.8%)		354 (94.9%)	19 (5.1%)	
≥14%	20 (80.0%)	5 (20.0%)		21 (84%)	4 (16.0%)	
Molecular subtype			**<0.001**†§			**<0.001**‡§
Luminal A	248 (95.4%)	12 (4.6%)		253 (97.7%)	6 (2.3%)	
Luminal B	24 (85.7%)	4 (14.3%)		26 (86.7%)	4 (13.3%)	
HER2-positive	26 (89.7%)	3 (10.3%)		29 (100%)	0 (0%)	
TNBC	64 (81.0%)	15 (19.0%)		67 (83.8%)	13 (16.2%)	

Bold indicated p<0.05 is statistically significant.
*Mann-Whitney U test.
†Pearson's χ^2 test.
‡Fisher's exact test.
§TN versus others.
ER, oestrogen receptor; HER2, human epidermal growth factor receptor 2; PDL1, programmed cell death-ligand 1; PgR, progesterone receptor; TILs, tumour-infiltrating lymphocytes; TNBC, triple-negative breast cancer.

Association of stromal TILs and PDL1 expression with patient prognosis

Overall, neither stromal TILs nor PDL1 expression were significantly associated with DFS or OS rates (table 4). However, in both G3 and ER-negative cases, the presence of more than 30% of stromal TILs was significantly associated with better DFS rates (94.26 vs 76.36; p=0.045 and 93.01 vs 68.02; p=0.044, respectively). Additionally, also in G3 BC cases, PDL1 expression was significantly associated with improved DFS (96.79 vs 77.39; p=0.043). In vimentin-positive BC cases, increased stromal TILs were also significantly associated with better DFS and OS rates (110.18 vs 77.35; p=0.037 and 113.36 vs 85.39; p=0.047, respectively) (see online supplementary table S3).

Table 3 Association between basal cell markers, CSC markers and EMT markers with stromal TILs and PDL1 expression in invasive breast cancer (first cohort)

	Stromal TILs			PDL1 expression		
	Absent/slight	Moderate/marked	p Value	Negative	Positive	p Value
Basal cell markers						
CK5			**<0.001***			**<0.001***
Negative	317 (93.8%)	21 (6.2%)		327 (96.5%)	12 (3.5%)	
Positive	45 (77.6%)	13 (22.4%)		48 (81.4%)	11 (18.6%)	
CK14			**0.005***			**0.031***
Negative	347 (92.5%)	28 (7.5%)		357 (94.9%)	19 (5.1%)	
Positive	15 (71.4%)	6 (28.6%)		18 (81.8%)	4 (18.2%)	
EGFR			**0.021***			**0.022***
Negative	347 (92.3%)	29 (7.7%)		359 (95.0%)	19 (5.0%)	
Positive	15 (75.0%)	5 (25.0%)		16 (80.0%)	4 (20.0%)	
P-cadherin			**0.007†**			**<0.001†**
Negative	278 (93.6%)	19 (6.4%)		289 (96.7%)	10 (3.3%)	
Positive	84 (84.9%)	15 (15.1%)		86 (86.9%)	13 (13.1%)	
CSC markers						
$CD44^{+}/CD24^{-/low}$			0.165†			**0.044†**
Negative	204 (93.2%)	15 (6.8%)		210 (96.3%)	8 (3.7%)	
Positive	157 (89.2%)	19 (10.8%)		163 (91.6%)	15 (8.4%)	
CD49f			0.251*			**0.011***
Negative	318 (91.9%)	28 (8.1%)		330 (95.4%)	16 (4.6%)	
Positive	38 (86.4%)	6 (13.6%)		39 (84.8%)	7 (15.2%)	
ALDH1			**0.014***			**0.033***
Negative	354 (92.2%)	30 (7.8%)		365 (94.8%)	20 (5.2%)	
Positive	8 (66.7%)	4 (33.3%)		10 (76.9%)	3 (23.1%)	
EMT markers						
E-cadherin			0.708*			0.628*
Negative/low	21 (95.5%)	1 (4.5%)		22 (100%)	0 (0%)	
Positive	339 (91.1%)	33 (8.9%)		352 (93.9%)	23 (6.1%)	
Vimentin			**0.008†**			**0.036***
Negative	307 (93.0%)	23 (7.0%)		316 (95.5%)	15 (4.5%)	
Positive	53 (82.8%)	11 (17.2%)		57 (87.7%)	8 (12.3%)	

Bold indicated p<0.05 is statistically significant.
*Fisher's exact test.
†Pearson's χ^2 test.
CSC, cancer stem cell; EMT, epithelial–mesenchymal transition; PDL1, programmed cell death-ligand 1; TILs, tumour-infiltrating lymphocytes.

Table 4 Association between stromal TILs and PDL1 expression in invasive breast cancer (first cohort)

	Stromal TILs			PDL1 expression		
	Absent/slight	Moderate/marked	p Value	Negative	Positive	p Value
PDL1 expression			**<0.001***			NA
Negative	380 (94.8%)	21 (5.2%)		–	–	
Positive	10 (35.7%)	18 (64.3%)		–	–	
DFS (mean of months)	86.5	97.8	0.087†	87.5	92.3	0.327†
OS (mean of months)	91.7	99.8	0.108†	92.7	92.9	0.568†

Bold indicated p<0.05 is statistically significant.
*Fisher's exact test.
†Kaplan-Meier method/log-rank p value.
DFS, disease-free-survival; NA, not applicable; OS, overall survival; PDL1, programmed cell death-ligand 1; TILs, tumour-infiltrating lymphocytes.

However, in multivariate analysis, only increased stromal TILs in vimentin-positive BC were independently associated with better DFS and OS (HR=0.10, p=0.017 and HR=0.06, p=0.019, respectively). Interestingly, also within vimentin-positive cases, PDL1 expression was, in contrast, significantly associated with a decreased OS (HR=7.12, p=0.018) (see online supplementary table S4 and figure 2).

Concerning molecular subtypes, we observed that moderate to marked stromal TILs were associated with better DFS (100.4 vs 72.6) and OS (103.8 vs 79.9) rates (p=0.096 and p=0.080, respectively) in TNBC, although without reaching statistical significance (see online supplementary table S5).

Stromal TILs and PDL1 expression in matched in situ and invasive carcinomas

When stromal TILs and PDL1 expression were compared between DCIS and IBC, a similar distribution was found (TILs—13.0% vs 13.8%, p=0.911; PDL1 expression—8.7% vs 13.8%, p=0.399, respectively), as actually happened for all the other characteristics previously evaluated (figure 3, tables 5 and online supplementary table S2).

Validating the results found in the first cohort, the presence of moderate to marked stromal TILs was significantly increased in G3 (26.9%; p=0.001), in TNBC subtype (25%; p=0.023) and in cases with PDL1 expression (63.6%; p<0.001) (see online supplementary table S6). We also observed an increase of stromal TILs in ER-negative cases and in cases with high expression of Ki67 (20%; p=0.059 and 20%; p=0.057, respectively). Additionally, we found a similar association of increased stromal TILs and HER2-positive subtype (25%; p=0.023). In DCIS, moderate to marked stromal TILs were enriched in G3 (23.5%; p=0.071), TNBC and HER2-positive subtypes (20% and 40%, respectively; p=0.073) and in cases with PDL1 expression (50%; p=0.077), although without reaching statistical significance.

Concerning PDL1 expression, it was significantly associated with G3 (26.9%; p=0.005) and all molecular subtypes, except luminal A (0%; p=0.005) (see online supplementary table S7). Interestingly, we also observed PDL1 expression enriched in cases with high expression of Ki67 (30%; p=0.061), without reaching statistical significance.

DISCUSSION

Our study confirms that increased stromal TILs are present in a minority of IBC cases and that are already present in in situ stages, as previously shown.[10 25] Although PDL1 expression has been reported in the literature from 20% to nearly 60% of the cases, we only observed in a small proportion of IBCs and, for the first time, even in DCIS cells.[26–30]

In IBC, increased stromal TILs and PDL1 expression were associated with each other and with G3 and TNBC subtype, with statistical significance in both cohorts, which also validates previous reports.[25 27 28] The association of increased stromal TILs and PDL1 expression with the evaluated basal cell markers also reinforces the relationship with the TNBC molecular subtype.

Stromal TILs include several types of T and B lymphoid cells in different proportions with distinct associations with clinical outcome that we did not discriminate in our study. For instance, cytotoxic (CD8+) T cells are significantly increased in high-grade, ER-negative BC and BC with increased proliferative activity, as well as associated with improved clinical outcome.[31–33] On the other side, immunosuppressive (CD4+/FOXP3+) T cells have been shown to be associated with worse prognosis in IBC and even with increased risk of relapse in DCIS.[34–36] These data suggest that cytotoxic T cells are responsible for the antitumour immune activity and that immunosuppressive T cells can inhibit this response. Furthermore, B lymphocytes are also associated with higher histological grade, ER-negative cases and basal phenotype, as well as with better prognosis.[37] This indicates that humoral immune response, along with cell-mediated immune response, acts in convergence to achieve effective antitumour response. Based on this knowledge, it would be important to evaluate the clinical value of subtyping the composition of stromal TILs in BC and reveal the potential predictive role of these markers in response to immunotherapy. Nonetheless, it is remarkable that the quantification of stromal TILs alone, regardless of its specific subpopulation of lymphoid cells, has prognostic and predictive information.

Although the use of TMAs in this work could, eventually, miss quantify stromal TILs evaluation, several reports suggest that TMAs are a valid choice, as the majority of BC are not heterogeneous regarding stromal TILs distribution throughout the tumour.[23 26 32 38] While recent guidelines recommend the evaluation of TILs as a continuous variable, we were able to show that the cut-off values used in this work enclose clinical significance and may be used to easily categorise this parameter, possibly reducing the interobserver variability.

Concerning PDL1 expression, in addition to tumour cells, it has been reported that it can be also present in stromal TILs, as observed by us, specifically in CD4+/FOXP3− T cells.[28] However, PDL1 expression is not associated with T cell

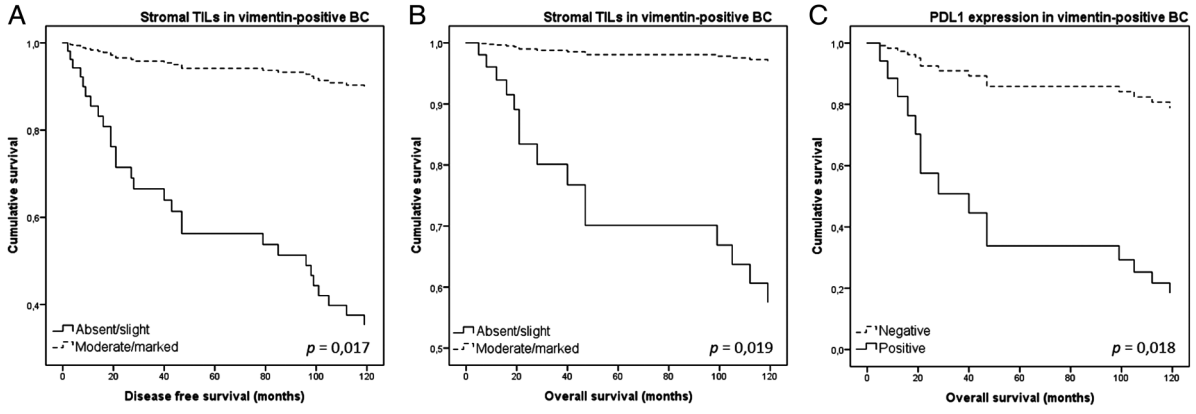

Figure 2 Survival curves showing the significant disease-free-survival and overall survival for patients with vimentin-positive breast cancer (BC) relatively to stromal tumour-infiltrating lymphocytes (TILs) (A and B) and programmed cell death-ligand 1 (PDL1) expression (C).

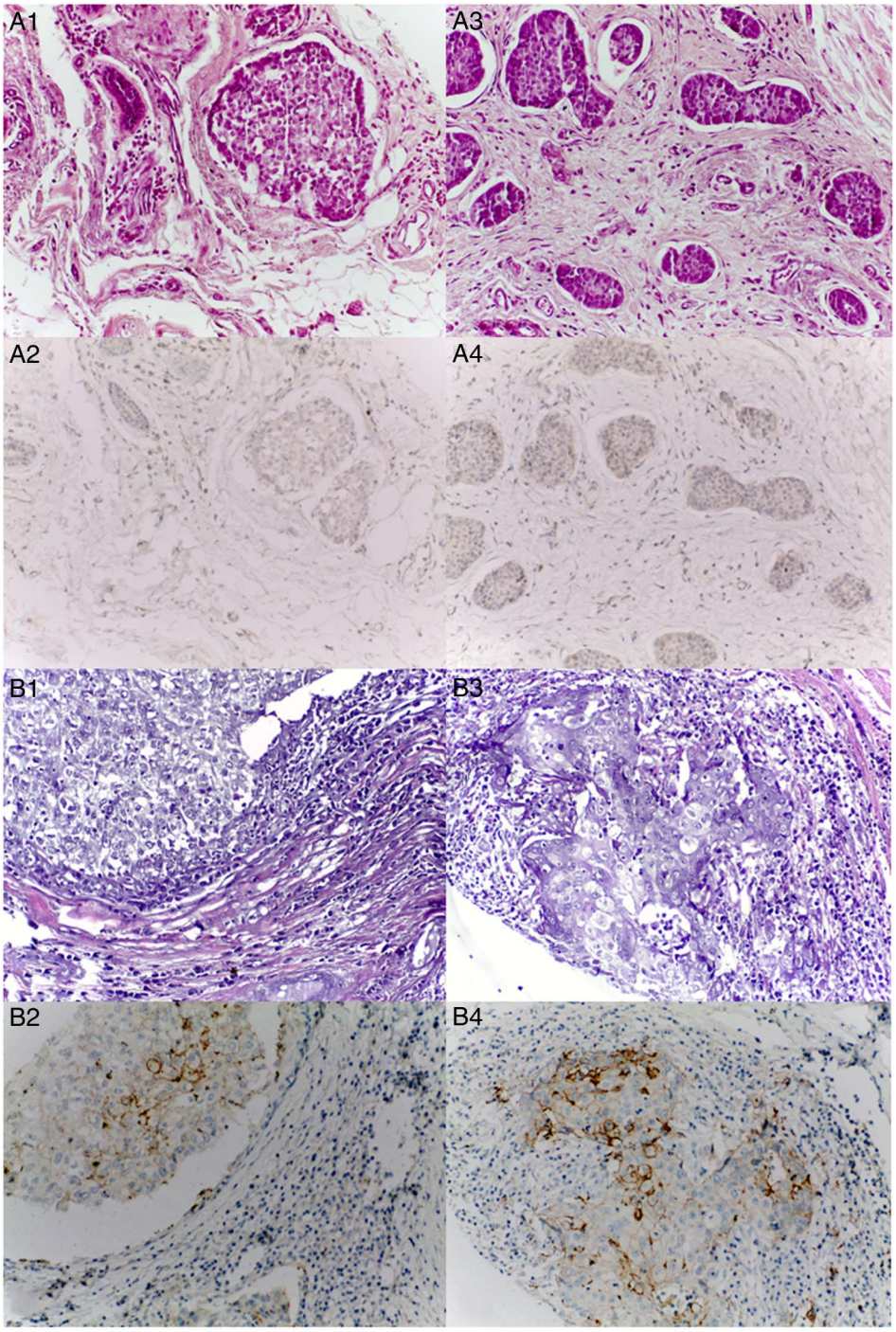

Figure 3 Stromal tumour-infiltrating lymphocytes (TILs) and programmed cell death-ligand 1 (PDL1) expression in pair-matched in situ and invasive breast cancer (IBC) (200×). (A) 1—ductal carcinoma in situ (DCIS) without stromal TILs (H&E); 2—PDL1-negative expression in DCIS; 3—IBC with few stromal TILs (H&E); 4—PDL1-negative expression in IBC. (B) 1—DCIS with increased stromal TILs (H&E); 2—PDL1 expression in DCIS; 3—IBC with increased stromal TILs (H&E); 3—PDL1 expression in IBC.

Table 5 Tumour-infiltrating lymphocytes and PDL1 expression in BC cases from the second cohort

	DCIS	Invasive carcinoma	p Value
Tumour-infiltrating lymphocytes			0.911*
Absent/slight	40 (87.0%)	69 (86.2%)	
Moderate/marked	6 (13.0%)	11 (13.8%)	
PDL1 expression			0.399*
Negative	42 (91.3%)	69 (86.2%)	
Positive	4 (8.7%)	11 (13.8%)	

*Pearson's χ^2 test.
BC, breast cancer; DCIS, ductal carcinoma in situ; PDL1, programmed cell death-ligand 1.

exhaustion markers, which means that PDL1 is only partially inhibiting T cells.[27] Nevertheless, this does not contradict the idea of blocking PDL1 in order to reactivate partially inhibited T cells and further increase the antitumour immune response.

Concerning DCIS, a similar trend in association between stromal TILs and PDL1 expression with histological grade and molecular subtype was also detected, suggesting that, as we previously reported, these features correlate well with the invasive counterpart.[22] Very recently, the expression of PDL1 has been described in about 80% of stromal TILs associated with DCIS cases, as well as an association with TNBC subtype.[39] In that work, there was no expression of PDL1 in DCIS cells, unlike our observation, in which we detected in both DCIS cells and associated stromal TILs.

The above-mentioned findings in DCIS samples indicate that the immune response to tumours exists even at early stages and that distinct molecular subtypes have different immunogenicity. The characterisation of immune checkpoint markers, in both invasive and DCIS cases, may result in potential targets for therapy, such as anti-PDL1 treatment. Moreover, we also report a similar distribution of stromal TILs and PDL1 expression between DCIS and IBC pair-matched cases. This means that stromal TILs and PDL1 expression might not be relevant for the progression from in situ to IBC, but instead represent a response to intrinsic characteristics of different molecular subtypes.

Finally, we observed that stromal TILs and PDL1 expression were associated with CSC markers and vimentin expression. Our results are expected because stromal TILs and PDL1 expression are associated with TNBC subtype, which in turn is associated with a higher proportion of cells with stem cell phenotype ($CD44^+/CD24^{-/low}$, CD49f and ALDH1) and EMT features, as shown by our group in the past.[21 40 41] In addition, as stated above, both stromal TILs and PDL1 expression were associated with basal cell markers, including P-cadherin, which was previously demonstrated by our group to be upregulated in basal-like subtype and associated with stem cell properties in BC cell lines.[40–43]

Although the association of stromal TILs with stem cell phenotype has been previously reported, as far as we know, this is the first time that PDL1 expression in BC cases is associated with CSC markers ($CD44^+/CD24^{-/low}$, CD49f and ALDH1), which is probably related to the close relationship with stromal TILs and TNBC subtype.[31] However, we observed an association of PDL1 expression with all CSC markers, while stromal TILs were only associated with ALDH1. This probably means a potential role of CSCs in the activation of PDL1 expression, both in tumour cells and stromal TILs, directly regulating the antitumoural immune response.

Despite the association of stromal TILs with CSC markers, the final effect in BC tissue is a protective one, given the fact that, in vimentin-positive BC, the presence of more than 30% of stromal TILs was independently associated with increased DFS and OS rates, reflecting an active antitumour immune response with an impact on patient outcome. Although contradictory information has been published regarding PDL1 expression and BC prognosis, in theory, the expression of PDL1 in BC should have a deleterious effect on prognosis, given the inhibitory effect of PDL1 in the antitumour immune response.[30] We also showed that in vimentin-positive BC, PDL1 expression is independently associated with worse OS. Although it has been reported that PDL1 expression can be related with better survival rates in BC, it is probably due to the close association of PDL1 with the presence of increased stromal TILs, the true effectors against tumour cells.[27 44] Finally, because IBC with mesenchymal traits is strongly associated with TNBC, a subtype associated with worse prognosis and limited treatment strategies, immunological modulators may represent an alternative approach to improve clinical outcome of these patients.[11 30 45]

In conclusion, we have confirmed the association of stromal TILs and PDL1 expression with aggressive BC (G3 and TNBC/basal subtype) and that both are already expressed in in situ stages. We also showed that increased stromal TILs and PDL1 expression are associated with clinical outcome in BC with vimentin expression. Notably, we describe a close relationship between CSC markers with stromal TILs, and for the first time, with PDL1 expression.

Take home messages

- Increased stromal tumour-infiltrating lymphocytes (TILs) are significantly associated with PDL1 expression in breast cancer (BC).
- PDL1 expression is present in in situ BC lesions.
- PDL1 expression is associated with cancer stem cell markers.
- Stromal TILs and PDL1 expression are independent prognostic markers in vimentin-positive BC cases.

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Prognostic value of stromal tumour infiltrating lymphocytes and programmed cell death-ligand 1 expression in breast cancer

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Notes